ORIGINAL ARTICLE

Cleavage pattern, gastrulation, and neurulation in the appendicularian, *Oikopleura dioica*

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Abstract The appendicularian, *Oikopleura dioica* is a chordate. Its life cycle is extremely short—approximately 5 days—and its tadpole shape with a beating tail is retained throughout entire life. The tadpole hatches after 3 h of development at 20°C. Here, we describe the cleavage pattern and morphogenetic cell movements during gastrulation and neurulation. Cleavage showed an invariant pattern. It is basically bilateral but also shows various minor left–right asymmetries starting from the four-cell stage. We observed two rounds of unequal cleavage of the posterior-vegetal B-line cells at the posterior pole. The nature of the unequal cleavages is reminiscent of those in ascidian embryos and suggests the presence of a centrosome-attracting body, a special subcellular structure at the posterior pole. The representation of the cell division pattern in this report will aid the identification of each cell, a prerequisite for clarifying the gene expression patterns in early embryos. Gastrulation started as early as the 32-cell stage and progressed in three phases. By the end of the second phase at the 64-cell stage, every vegetal cell had ingressed into the embryo, and animal cells had covered the entire embryo by epiboly. There was no archenteron formation. In the anterior region, eight A-line cells were aligned as a 2×4 array along the anterior–posterior axis and become internalized during the 64-cell stage. This

process was considered to correspond to neurulation. The simple and accelerated development of Oikopleura, nevertheless giving rise to a conserved chordate body plan, is advantageous for studying developmental mechanisms using molecular and genetic approaches and makes this animal the simplest model organism in the phylum Chordata.

Keywords Cleavage pattern · Gastrulation · Neurulation · Appendicularian · *Oikopleura dioica*

Introduction

Appendicularians are pelagic or planktonic tunicates (urochordates), and retain a swimming tadpole shape throughout their life. For this reason, they are sometimes referred to as larvaceans. Together with ascidians and thaliaceans, they comprise the sister group or closest relatives of vertebrates, appendicularians being the most basal among these tunicates (Delsuc et al. 2006; Bourlat et al. 2006; Swalla et al. 2000), although the phylogenetic position of the appendicularians among chordates is still controversial (Stach and Turbeville 2005). They represent the basic body plan of chordates and include features such as a tail with a notochord and dorsal neural tube, an endostyle, and a gill aperture. The structure of the adult body is relatively simple and consists of a small number of cells; for example, there are only 20 notochord cells at hatching stage and 20 non-fused muscle cells even in a mature adult (Nishino et al. 2000, 2001; Bassham and Postlethwait 2000; reviewed by Fenaux 1998a; Nishino and Satoh 2001).

Oikopleura dioica is an emerging model animal for use in studies of development. It has a miniature genome of 65 Mb, which is the smallest ever found in a chordate (Seo et al. 2001). Its life cycle is extremely short—about 5 days

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at 20°C (Fenaux 1998b)—and it can be maintained in the laboratory over many generations. These advantages make this organism a suitable experimental model animal, in which genetic approaches, transgenesis, and mutagenesis would be feasible, as in *Drosophila* and *Caenorhabditis elegans*. Recently, several groups started molecular and comparative approaches for studying the embryogenesis of this animal (e.g., Nishino et al. 2000, 2001; Bassham and Postlethwait 2000; Thompson et al. 2001; Seo et al. 2004; Cañestro and Postlethwait 2007).

To understand the mechanisms involved in embryogenesis, descriptions of the cleavage pattern, cell lineages, and morphogenetic movements are indispensable. Detailed descriptions of cleavage patterns and cell lineages are especially important for animal embryos that show invariant and determinative cleavage patterns, as is the case for *C. elegans* (Sulston et al. 1893) and ascidians (Conklin 1905; Nishida 1987). Precise and unambiguous identification of each blastomere is essential for analyses of gene expression in early embryos. After the cleavage stage, gastrulation, the first extensive morphogenetic movement begins. The cellular and molecular events of this process have been intensively investigated in various animals (Stern 2004). Despite the fact that *Oikopleura* has a small number of embryonic cells and can be cultured readily in the laboratory, only a few reports describing its embryogenesis have appeared so far (Delsman 1910; Fenaux 1998b; Nishino and Satoh 2001). Among these descriptions, Delsman's (1910) is remarkably detailed and comparable to Conklin's (1905) monumental paper on ascidian embryogenesis. Delsman investigated the cleavage pattern of *Oikopleura dioica* in amazing detail using embryos at various stages collected directly from the ocean with a plankton net. He succeeded in naming each blastomere during the cleavage and gastrulation stages.

In the present study, we investigated the embryonic cleavage pattern of *Oikopleura dioica* using modern techniques, such as time-lapse video and Nomarski and confocal microscopy, to confirm previous descriptions and to provide an easily accessible data source for embryos up to the gastrula stage. Our findings confirm that Delsman's description was substantially accurate, with a single exception in that the posterior-most blastomeres appear to be generated by sequential unequal cell divisions.

Materials and methods

Appendicularian culture

Laboratory culture of *Oikopleura dioica* was started with specimens collected near the Misaki Marine Biological Station on the Miura peninsula and the Seto Marine Biological Laboratory on the Kii peninsula, Japan. The animals were cultured basically according to Chioda et al. (2002). They were cultured in artificial seawater (Rohto-Marine, Rei-Sea Co, Tokyo, Japan) in 5-l plastic beakers under constant stirring with an acrylic paddle ($0.2 \times 7 \times 20$ cm, 15 rpm) at 20°C. Approximately 10 g of activated charcoal was added to each beaker. The animal's life cycle was 5 days. Cultures were diluted 1:5 on day 1, and then 100 animals were transferred to clean artificial seawater on day 3 by pipetting and allowed to naturally spawn on day 5. The animals were fed with a cocktail of live and various-sized algal strains, including *Isochrysis galbana* (approximately 4 μm, 3,000 cells/ml), *Chaetoceros calcitrans* (3–6 μm, 3,000 cells/ml), *Synecococcus* sp. (1.5 μm, 4.0 ml of the culture/5 l), and *Rhinomonas reticulata* (12 μm, 1,000 cells/ml) on days 1 and 2, and then at double those concentrations from days 3 to 5.

Embryos

Mature adult males and females were separately transferred one by one to small dishes and allowed to spawn naturally. Eggs were artificially fertilized with diluted sperm. Embryos were then left to develop at 13–20°C. Tailed larvae hatched 6 h after fertilization at 13°C and 3 h after at 20°C. Each event described in this report was observed three times or more in independent experiments. The nomenclature for the blastomeres is according to Delsman (1910).

Cytoplasmic actin staining

To visualize the cell membrane, embryos were stained with phalloidin basically according to Munro and Odell (2002). Embryos were fixed with 4% paraformaldehyde in 90 mM Pipes buffer (pH 6.9), 45 mM diaminoethanetetraacetic acid (EDTA), and 360 mM sucrose for 30 min at room temperature. After washing with phosphate buffered saline (PBS) containing 0.2% Triton X-100 (PBST), they were stained with 5 unit/ml Alexa Fluor 488 phalloidin in PBST for 1 h at room temperature then washed with PBST. Specimens were mounted in 80% glycerol and observed.

Microscopy

Developing embryos were mounted in a hollow glass slide, observed with Nomarski optics, and photographed. Time-lapse video recordings were carried out with a video camera (Hamamatsu Photonics C2400, Hamamatsu, Japan). Acquired image sequences were digitized with an analog-to-digital converter and further processed with QuickTime Pro and Image J (NIH). Fixed and stained embryos were observed with a fluorescence microscope (Olympus BX61) and an Axioskop (Carl Zeiss) confocal microscope

equipped with a CSU10 confocal unit (Yokogawa). In confocal analysis, Z-stack images were acquired with IPLab (Solution Systems, Funabashi, Japan), then 3-dimensional images and movies were generated with VoxBlast (Solution Systems) and DeltaViewer software (Nara Women's University).

Results

Embryogenesis: up to hatching

Unfertilized eggs are arrested at the first meiotic metaphase I (Ganot et al. 2007). There is no accessory cell inside or outside the vitelline membrane. Just after fertilization (30 s), the surface of the egg became rough. This is the first sign of fertilization. After fertilization, no expansion of the narrow perivitelline space was observed. Emission of two polar bodies in 15 min was followed by the first cleavage at 35 min at 13°C (Fig. 1 and Supplemental movie S1). Early cleavages take place every 15 min. Developmental time became roughly half when embryos were cultured at 20°C.

At 2 h of development, gastrulation started at the 32-cell stage. Every vegetal blastomere was internalized during gastrulation. The tailbud stage was attained at 4 h, then the tail elongated and the embryo became bent ventrally. Notochord cells aligned in a single row became evident at 4.5 h. Tailed larvae hatched from the vitelline membrane at 6 h (Fig. 1). There was no obvious structure in the head and trunk regions, but the larvae moved with occasional tail beating. Organogenesis in the head and trunk regions was completed by 18.5 h, when the tail quickly bends anteriorly (so-celled metamorphosis or tail shift in O. dioica; Fenaux 1998b).

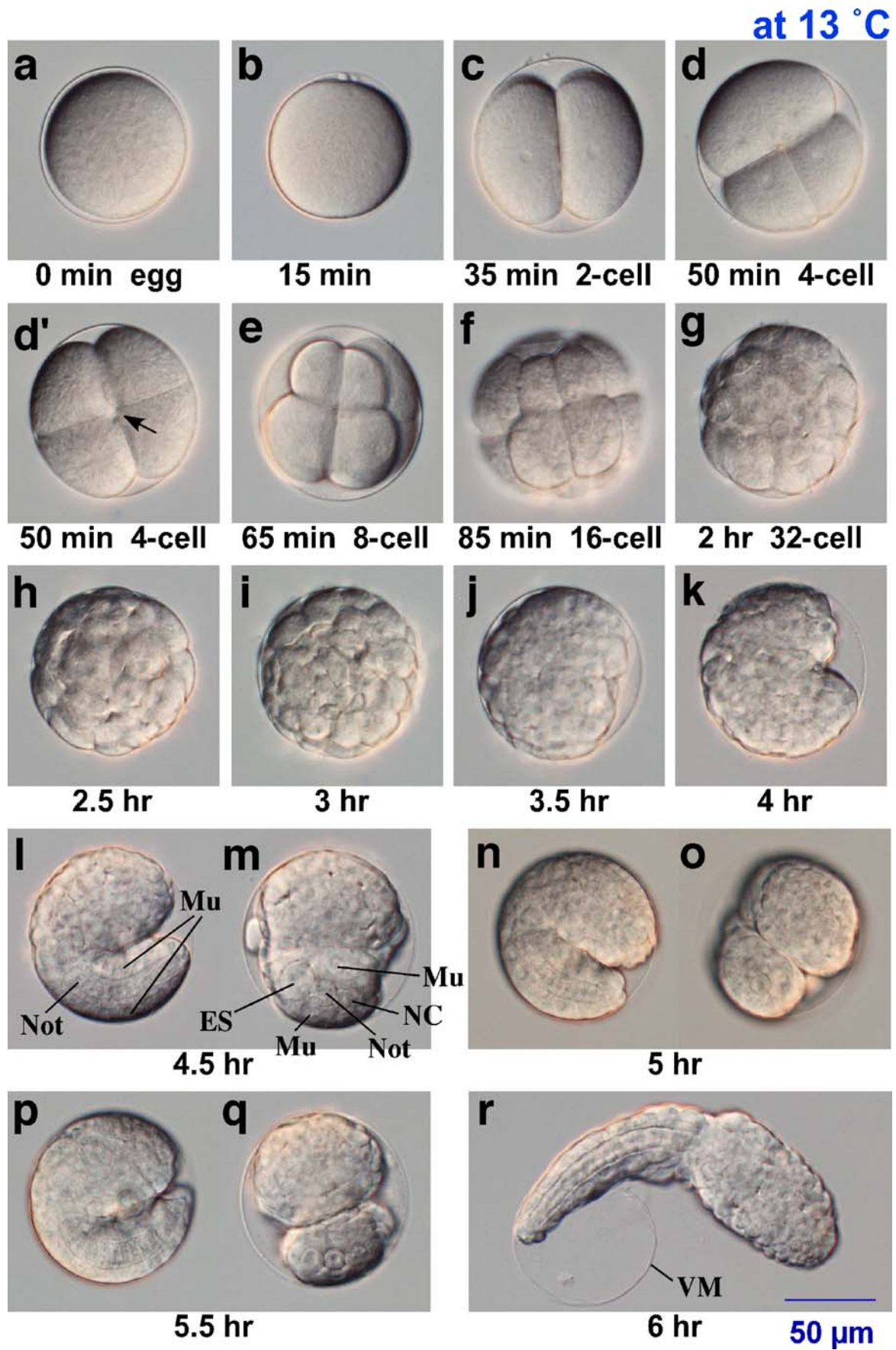Cleavage pattern: up to the 32-cell stage

Cleavage showed an invariant pattern among individuals. The first cleavage furrow formed close to the position of the polar bodies, and the second furrow also did so. There was no size difference between the blastomeres at this stage. The first cleavage generates the left and right blastomeres (Fig. 1a–d' and Supplemental movie S1), and the second cleavage plane divides the anterior and posterior blastomeres, as in ascidian embryos (Delsman 1910; Nishida 2005), which are equivalent to the dorsal and ventral blastomeres in amphibian embryos, respectively (See Nishida (2005) for differences in conventional definition of the anterior–posterior and dorsal–ventral axes in tunicates and amphibian embryos).

It is noteworthy that in most four-cell embryos, two opposing blastomeres were in contact with each other at the

animal pole, and the other two blastomeres were in contact at the vegetal pole. The size of this contact area at the poles varied from embryo to embryo (Fig. 1d,d', arrow). It is likely that the second cleavage furrows forming in the left and right blastomeres of the two-cell embryo are not in the same plane and slightly tilted relative to each other. At the four-cell stage, the anterior–posterior and left–right axes that are perpendicular to the animal–vegetal axis are not morphologically recognizable. However, temporal retrospective observations of sequential photographs of the same embryos and time-lapse videos revealed that the anterior-left and posterior-right blastomeres of the four-cell embryo are invariably formed slightly closer to the vegetal pole, where they are in contact with each other. On the other hand, the anterior-right and posterior-left blastomeres are in contact at the animal pole. This is the first sign of invariant left–right asymmetry in the O. dioica embryo. The contacts between blastomeres at the animal and vegetal poles were stable and still evident in later-stage embryos, namely, contact at the animal pole of the right a and left b blastomeres at the eight-cell stage, right a1 and left b1 at the 16-cell stage, and right a11 and left b11 at the 32-cell stage (see Figs. 2, 3). Similarly, the left A and right B blastomeres at the eight-cell stage, left A2 and right B2 at the 16-cell stage, and left A22 and right B22 at the 32-cell stage were in contact at the vegetal pole.

The third cleavage occurred horizontally, then four smaller vegetal blastomeres and four larger animal blastomeres were generated. As described later, all the vegetal blastomere descendants were internalized during gastrulation. From this stage onward, the anterior–posterior and left–right axes as well as the identity of each blastomere are recognizable (Fig. 2). We followed the blastomere nomenclature used by Delsman (1910). At the eight-cell stage, each blastomere is named according to the ascidian system (Conklin 1905) as Delsman did. Animal cells are indicated by a lower case letter and vegetal cells by a capital. Anterior cells are named "a" and "A," and posterior cells are named "b" and "B." The cells of the right half when viewed from the vegetal pole (future dorsal side) with the anterior pole upward are labeled by underlining. Note that the vegetal pole (future dorsal side) is down in the anterior, posterior, right, and left views in Fig. 2. The size of animal (a, b, a, b) blastomeres was almost the same, while in the vegetal hemisphere, the anterior "A" blastomere pair were significantly smaller than the posterior "B" blastomere pair.

At the next cell division leading to the 16-cell stage, every vegetal blastomere divided along the anterior–posterior axis, while every animal blastomere divided along the left–right axis (Fig. 3a–d). Thus, the 16-cell embryo consisted of a U-shaped (2×4 cells) vegetal and an upside down U-shaped (2×4 cells) animal hemisphere. From this stage, Delsman (1910) applied the nomenclature system used in annelid and

at 13 ˚C

a 0 min egg

b 15 min

c 35 min 2-cell

d 50 min 4-cell

d' 50 min 4-cell

e 65 min 8-cell

f 85 min 16-cell

g 2 hr 32-cell

h 2.5 hr

i 3 hr

j 3.5 hr

k 4 hr

l Mu Not 4.5 hr

m ES Mu Mu NC Not
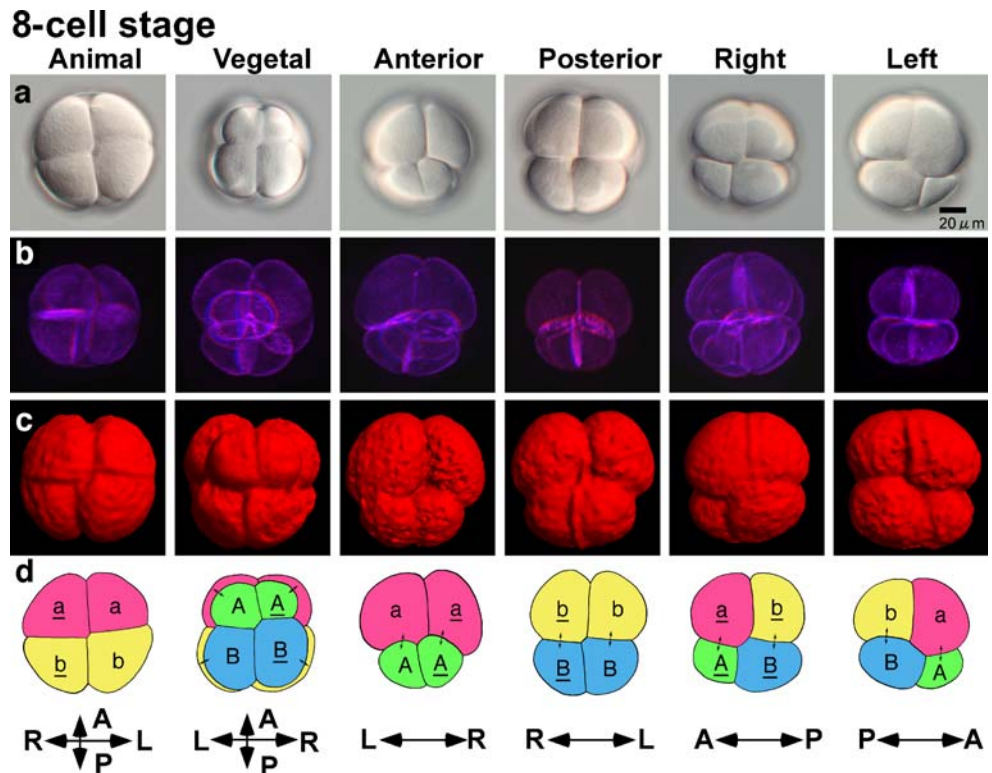
n

o 5 hr

p

q 5.5 hr

r VM 6 hr 50 μm

Fig. 1 Embryogenesis of *Oikopleura dioica*. Developmental times and stages are indicated below each photo. **a** Unfertilized egg; it is approximately 80 μm in diameter. **b** Fertilized egg after emission of the second polar body. **c** The 2-cell stage. **d, d'** Two examples of four-cell embryos. Note the contact between two opposing blastomeres at the animal or vegetal pole (*arrow*) in (**d'**). **e–g** 8-, 16-, and 32-cell embryos. **h–k** Late–gastrula to tailbud stages; photos were taken every 30 min. **l–q** Right and ventral views at each stage during tail elongation. In optical sections of the tail, muscle (*Mu*), notochord (*Not*), nerve cord (*NC*), and endodermal strand (*ES*) are visible. The tail has rotated counter-clockwise by 90° when viewed from the posterior direction with the nerve cord on the left side. **r** Hatching tadpole larva. See also "Supplemental movie S1"

mollusk embryos. All the descendant cells of a given blastomere in the eight-cell embryo are indicated with the character of the blastomere. After each division, a figure 1 or 2 is added, 1 for the daughter cell closer to the animal pole. For instance, blastomere "A" divides into A1 and A2. Then the A1 blastomere divides into A11 and A12, and the A2 divides into A21 and A22. In most cells, the fourth division was equal in size except for the B posterior-vegetal blastomeres. Division of B cells was markedly unequal, generating smaller B1 cells posteriorly. It was also noted that the midline became intricate, especially in the vegetal hemisphere. This is because the A1 cell pair shifted rightward and the B1 cell pair shifted leftward, in addition to contact between A2 and B2 cells at the vegetal pole mentioned previously (Fig. 2d, vegetal view).

From the 16-to the 32-cell stage, most of the animal cells divided perpendicularly to the previous division, namely along the anterior–posterior axis, except for the b2 cell pair.
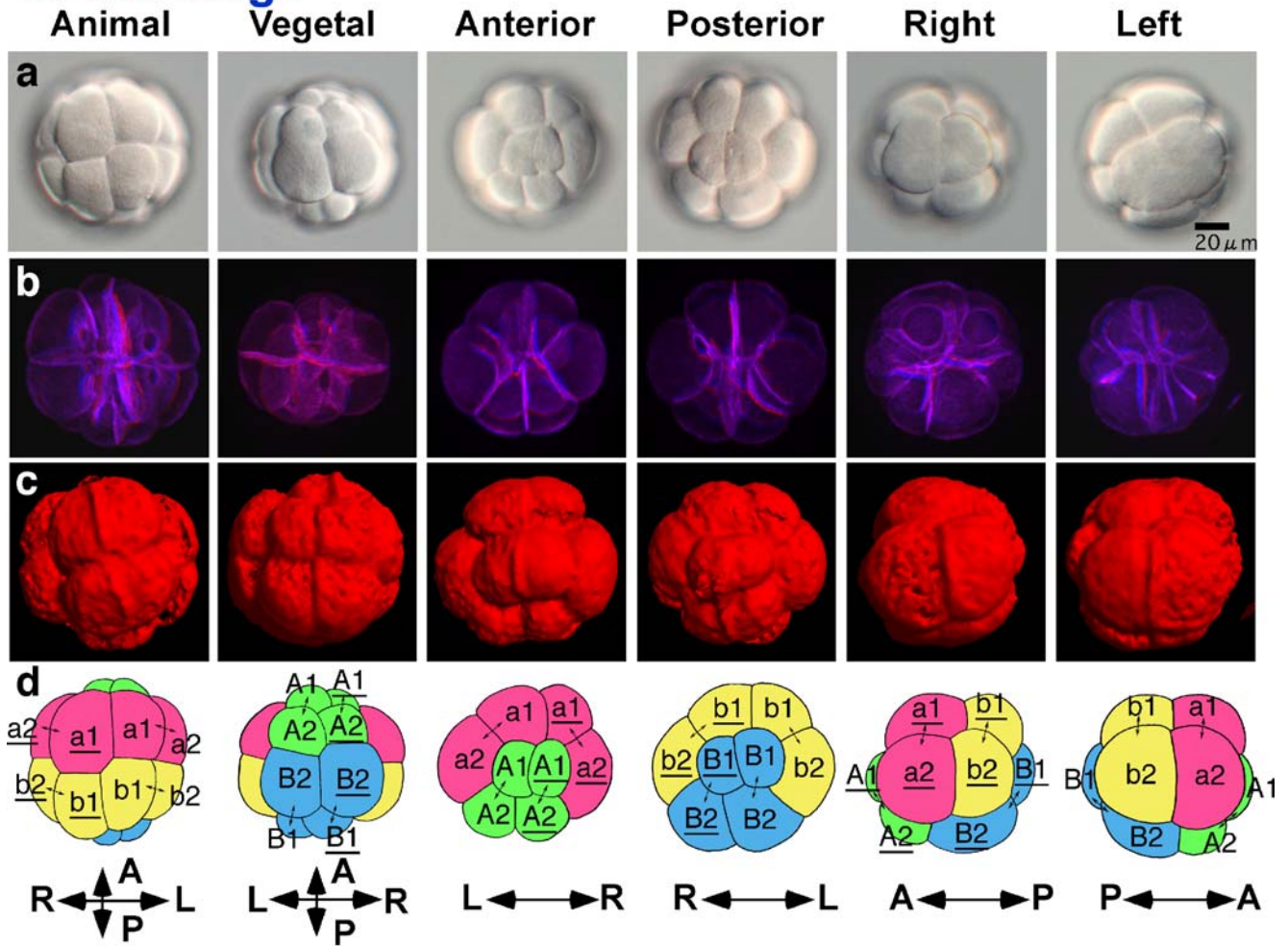
They then divided along the left–right axis again, generating b21 and b22 cell pairs (Fig. 3e,f). In the vegetal hemisphere, four cells situated at the vegetal pole (A2 and B2 pairs) divided perpendicularly to the previous division, namely along the left–right axis (division of A2 slightly preceded that of B2; see Supplemental movie S3), while the anterior and posterior sister cells (A1 and B1 pairs) divided along the anterior–posterior axis again. However, precisely speaking, the division of the right A2 and right B2 was lateral, whereas that of the left A2 and left B2 was oblique, so that left-sided daughter cells (A21 and B21) were always formed more anteriorly (Fig. 3f, vegetal view). With regard to blastomere sizes, most of the fifth division was almost, if not perfectly, equal. The only one exception was the most posterior (B1) blastomeres that were the smaller ones formed by the previous unequal division. This blastomere pair divided quite unequally, generating larger B12 and smaller B11 situated at the posterior pole (Fig. 3f, posterior view, and Supplemental movie S2). The position of the tiny B11 cells well corresponds to Delsman's "mysterious" cells. Thus, two rounds of unequal division took place at the posterior region, generating smaller and smaller daughters at the posterior pole.
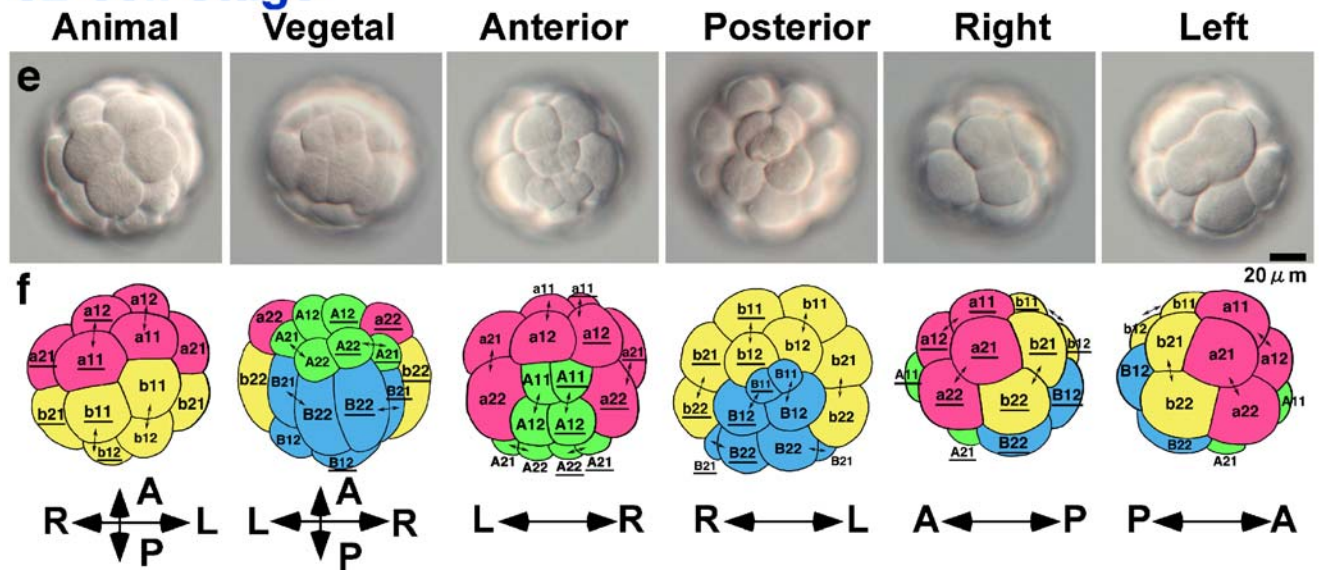
Gastrulation and neurulation: 32-cell stage onward

In *O. dioica*, gastrulation was initiated quite early and proceeded in three phases, the first starting at the 32-cell stage. In the first phase, the vegetal eight (4×2) cells ingressed (Figs. 4a–d, 5a–e, and Supplemental movie S3).

Fig. 2 Arrangement of the blastomeres at the eight-cell stage; embryos were viewed form various directions indicated at the *top*. The cleavage pattern was invariant among individuals. Orientations of embryos are indicated at the *bottom*. **a** Embryos observed with Nomarski optics; **b** 3-D representations of plasma membranes for red-blue-glasses. Images were constructed from confocal image stacks of embryos stained with Alexa Fluor 488 phalloidin. **c** 3-D representations of surface views; snapshots of corresponding angles of 3-D images generated with Delta-Viewer software. **d** Schematic representations; blastomere names are given. *Bars* connect sister blastomeres. Note that the vegetal pole (future dorsal side) is down in the anterior, posterior, right, and left views

16-cell stage

Animal	Vegetal	Anterior	Posterior	Right	Left

32-cell stage

| Animal	Vegetal	Anterior	Posterior	Right	Left

◀ **Fig. 3** Arrangement of the blastomeres at the 16- and 32-cell stages. Embryos were viewed from various directions indicated at the top. **a–d** The 16-cell stage. **e, f** The 32-cell stage; see legends for Fig. 2. The vegetal pole (future dorsal side) is down in the anterior, posterior, right, and left views. Note that two rounds of unequal cell division have occurred in B-line cells in the posterior views. See also "Supplemental movie S2" for these unequal cell divisions

Ingression of the lateral four cells (A21 and B21 cell pair) slightly preceded that of the medial four cells (A22 and B22 cell pair). After completion of ingression, the relative positions between these blastomeres were identical to those

of pre–gastrulation, namely, left-most cells (A21 and B21) always lying more anteriorly than the others (Fig. 5g). These eight cells divided along the animal–vegetal axis after ingression completion. Concomitantly to internalization of these cells, the cells that marginally flanked them before–gastrulation covered the vegetal surface (Figs. 4d green cells, 5d). There was no archenteron formation. The outline of the embryo remained spherical until tail bud formation. When ingression of these eight cells had been completed, the covering cells were arranged like an asterisk (Figs. 4d, 5f) at
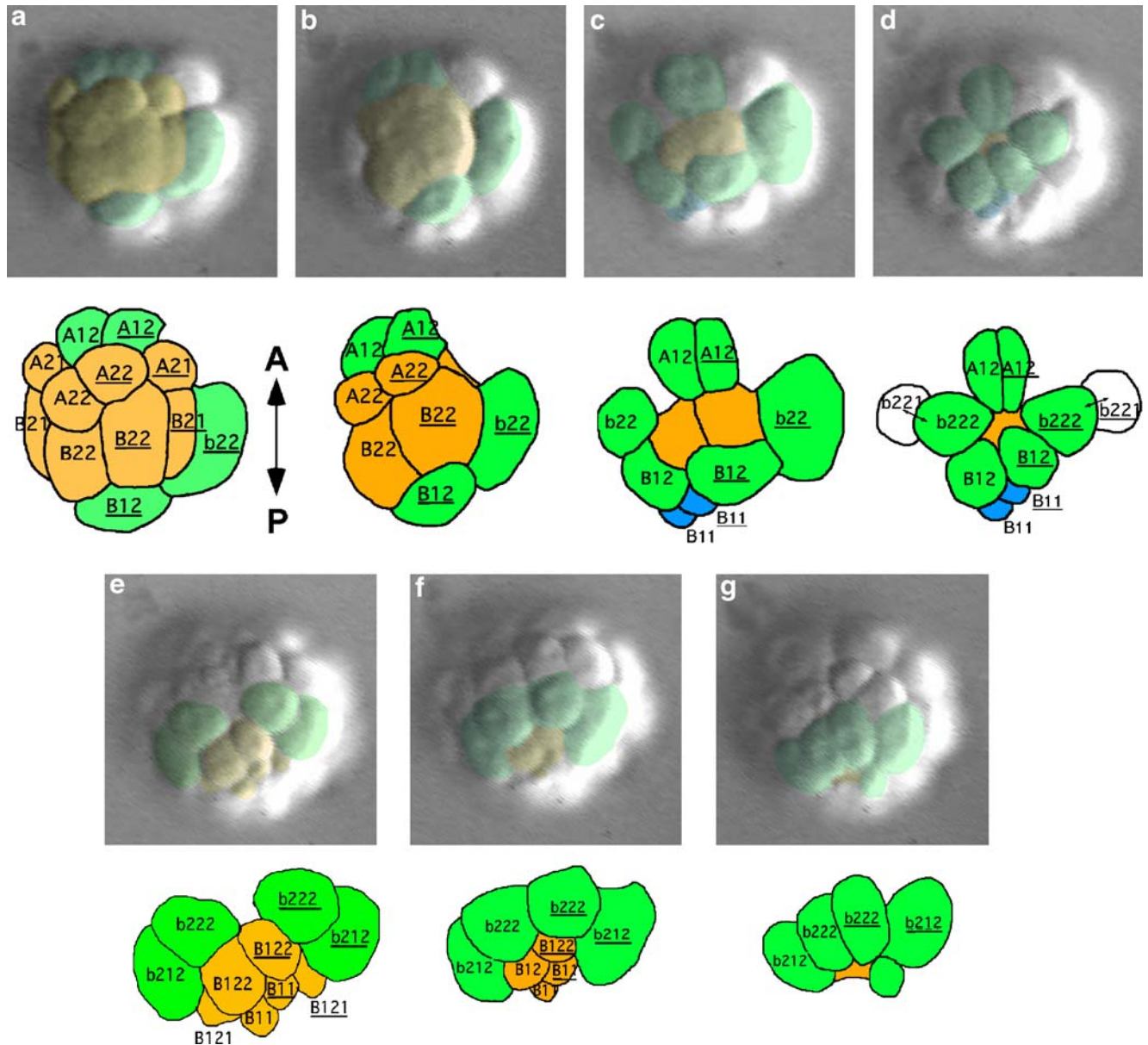
Fig. 4 Gastrulation. **a–d** The first phase; sequential images of time-lapse video taken from 105 to 122 min after fertilization at 13°C. Eight yellow-colored blastomeres at the vegetal pole are ingressing. The embryonic surface becomes covered by six green-colored blastomeres by epiboly. These six cells were arranged like an asterisk at the completion of ingression of the yellow cells. B11 cells, sister

cells of B12, generated by unequal division are shown in *blue*. Anterior is up. **e–g** Subsequent sequence showing the second phase of gastrulation from 122 to 145 min; the B12 cells have divided into B122 and B121 and are now colored yellow. Six yellow-colored cells are internalized sequentially in the order B121, B122, and B11. See corresponding "Supplemental movie S3"
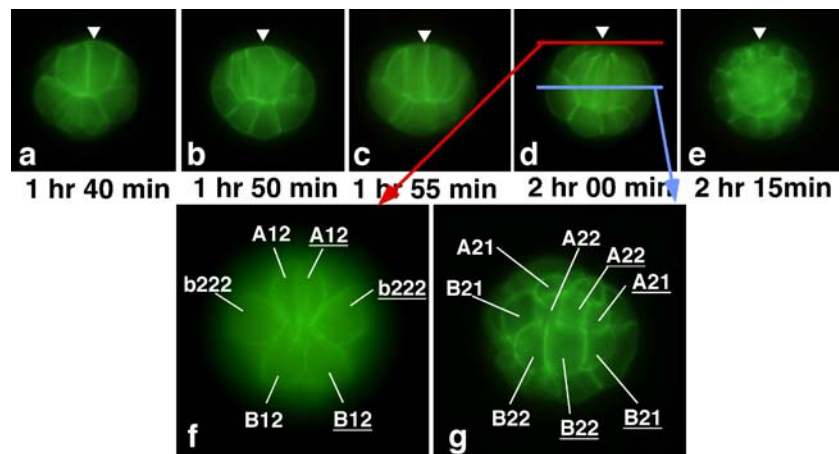
Fig. 5 Gastrulation. The cell membrane was visualized with Alexa Fluor 488 phalloidin. **a–e** Side views of embryos during the first phase. Vegetal pole is up and indicated by *white arrowheads*. **a** The 16-cell stage. **b–d** Vegetal cells are ingressing at the 32-cell stage. Note that the embryonic outline is kept almost spherical. **e** The 64-cell stage. **f** Surface view at the vegetal pole corresponding to the level indicated by the *red line* in (**d**). An asterisk-like arrangement of the six cells is visible. **g** Eight ingressed cells were observed at the level indicated by the *blue line* in (**d**). Note that the left A21 and B21 cells lie anteriorly relative to the other cells

the vegetal surface. The anterior arms were made up of right and left A12 cells, which were in lateral contact with each other. The lateral arms consisted of the b222 cell pair, and the posterior arms were the B12 cell pair.

In the second phase of gastrulation, the anterior and posterior arms (A12 and B12 cell pairs) ingressed. In the posterior region, B12 and its small sister B11 cells still faced the embryo surface after the first phase (Fig. 4d). The B12 cell pair that formed the posterior arms of the asterisk divided into anterior B122 and posterior B121. During the second phase, these blastomeres ingressed sequentially in the order B121, B122, and B11 (Fig. 4e–g and Supplemental movie 3). The b222 cell pair that formed the lateral arms of the asterisk came in close contact with each other and covered the surface of the ingressing cells.

In the anterior region, neurulation starts. The A11 and A12 cells face the surface after the first phase (Fig. 6a). The behavior of these cells is of particular interest, as they are the anterior and posterior (A–P) sister cells that were generated by the previous division along the A–P axis. These cells became elongated along the A–P axis and divided again along the same axis. Thus, eight (4×2) cells originated from an A1 cell pair. These eight cells were simultaneously internalized by ingression, and the laterally flanking cells (a221 and a222 cell pairs) covered the surface (Fig. 6 and Supplemental movie 4). This process seems to correspond to neural tube formation in other chordates, as discussed later. Thus, all the vegetal cells that originated from A and B cells of the eight-cell stage embryo were internalized. At this point, most of the cells have completed

Fig. 6 Neurulation. Sequential images of time-lapse video taken from 120 to 130 min after fertilization at 13°C. Anterior is at the bottom left, and the vegetal pole at the upper right. Four yellow-colored cells of A1 descendants in the anterior region (**a**) divide once along the anterior–posterior axis (**b**) and are then internalized (**c**). The surface is covered by green-colored cells. See corresponding "Supplemental movie S4"
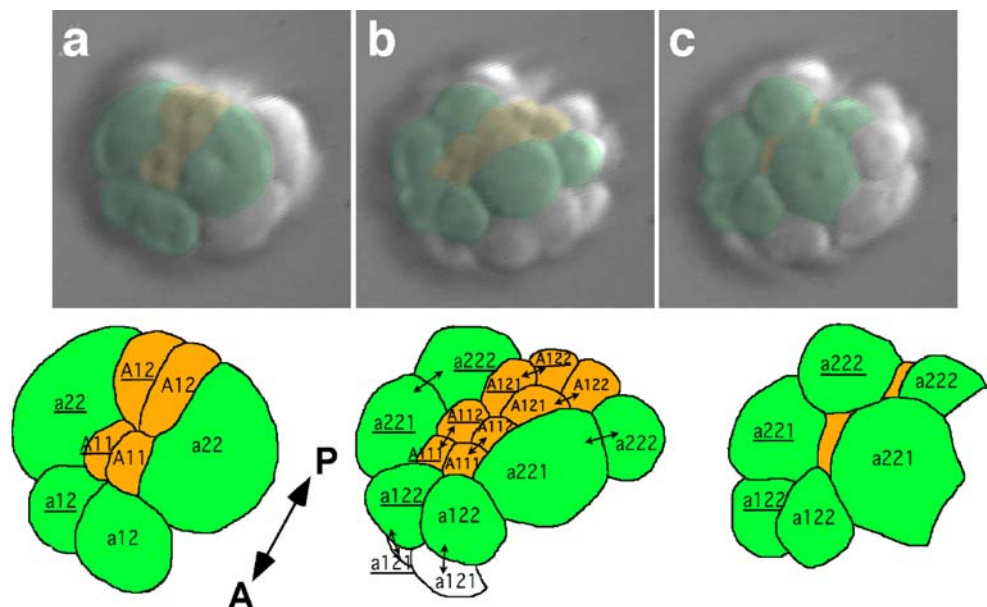
the sixth division and are named with one letter and three numbers (e.g., a222 and B212). Thus, the number of constituent cells at this stage is roughly 64, although the smallest cells at the posterior pole (B11) do not undergo cell division.

Recently, complete cell lineages have been traced up to the hatching stage by Stach and his colleagues (Freie Universität Berlin, personal communication). According to their data, the b222 and b221 pairs (Fig. 4d–g) that are daughter cells of the b22 pair of the 32-cell embryo are internalized in the posterior-lateral region, and the a222 pair (Fig. 6b,c) also ingress in the anterior-lateral region in the third phase. Thus, all cells forming the internal structures of the hatching larva complete their internalization.

Discussion

In this study, using modern techniques, we observed and described in detail the cleavage pattern and gastrulation–neurulation processes in *O. dioica*. We confirmed that the descriptions by Delsman (1910) were amazingly precise and accurate, except for one new finding of unequal division of B1 blastomeres, which will be discussed later. Reports focusing on *O. dioica* embryogenesis have recently been increasing because the genome of this animal was sequenced (Seo et al. 2001). To study early embryogenesis, it is essential to identify blastomeres that express specific genes. Detection of genes that are expressed in specific subsets of blastomeres has significantly advanced our understanding of early cell fate specification processes in ascidian embryos (Imai et al. 2006; reviewed in Nishida 2005). In *Oikopleura*, expression of *brachyury* and the *muscle actin* genes is initiated in notochord and muscle precursor blastomeres at the 32- and 64-cell stage, respectively (Nishino et al. 2000, 2001; Bassham and Postlethwait 2000). The cleavage pattern of *O. dioica* is somewhat complicated, as the pattern is roughly, but not precisely, bilateral. This makes it difficult to recognize the orientation of embryos as they lack a straight midline, unlike ascidian embryos. The representation of the cleavage pattern viewed from every direction in this report will aid the identification of each blastomere, which is a prerequisite for clarifying patterns of gene expression and labeling and tracing cell fates during development.

Left–right asymmetry

The *O. dioica* body shows several invariant left–right asymmetries. The digestive tract is not bilaterally symmetrical (Burighel et al. 2001), and the tail shows a remarkable 90-degree counter-clockwise rotation relative to the trunk when viewed from the posterior, with neural tube on the left side of the notochord. The rotation of the tail reflects the direction of the tail beat. In ascidians, amphioxus, and lower vertebrates, the tail beats in a left–right direction, while in larvaceans it beats in a dorso-ventral direction. Muscle cells are located in the dorsal and ventral parts of the tail, and the central nervous system is located on the left side of the tail. When the tail starts to form in tailbud embryos, this twisted positioning was already evident (Fig. 1l–q). Therefore, the left–right asymmetry starts early in embryogenesis.

The first sign of left–right asymmetry is recognizable at the four-cell stage. The anterior-left and posterior-right blastomeres of the four-cell embryo are invariably formed slightly closer to the vegetal pole and they are in contact with each other at the vegetal pole. The other two blastomeres are in contact at the animal pole. The contacts of their daughters at the poles were stable at least until the 32-cell stage. At the 16-cell stage, the A1 cell pair shifted rightward, while the B1 cell pair shifted leftward. These arrangements of blastomeres make the median plane intricate. In 32-cell embryos, the left A21 and B21 cells were always positioned more anteriorly than their sister cells and bilateral right counterparts. At the moment, it is unclear how these early events in the development of left–right asymmetry are related to the final left–right asymmetry of larval morphology. Tracing the cell fates of these blastomeres during later morphogenetic movements would shed light on this issue.

Unequal cell divisions at the posterior pole

Two rounds of unequal cell division were observed in the posterior-vegetal B-line blastomeres. In the second round, B1 blastomeres that reside at the posterior pole of the 16-cell embryo divided unequally into the larger B12 and the smaller B11. Delsman (1910) mentioned that the tiny cell (that he called the mysterious cell) is generated by division of the animal b12 cell, although he had some doubt about this. In the present study, it was clearly demonstrated by time-lapse video (Supplementary video S2) that this tiny cell pair is generated by unequal division of the B1 cell pair.

Thus, the cleavage pattern of the *O. dioica* embryo shows striking similarity with that of ascidians. In ascidians, three rounds of unequal division take place at the posterior pole of the vegetal hemisphere from the 8- to 64-cell stages. These unequal divisions always generate smaller daughter cells at the posterior pole and, as a result, most posterior cells become much smaller than the other cells (Conklin 1905; Nishida 1987). Finally, the posterior pole cells of the 64-cell-stage embryo are fated to become primordial germ cells in ascidians (Tomioka et al. 2002; Shirae-Kurabayashi et al. 2006). In *O. dioica*, we observed

two rounds of unequal divisions similarly occurring at the posterior pole starting from the B blastomeres of the eight-cell embryo. We are uncertain whether the resulting tiny B11 cell is a primordial germ cell, but comparison with ascidian development suggests that this is plausible.

The posterior blastomeres in ascidians have a special subcellular structure, the centrosome-attracting body (CAB; Hibino et al. 1998; Nishikata et al. 1999; Iseto and Nishida 1999; Negishi et al. 2007). During unequal cleavages, the microtubule bundle extending from the more posterior of the two centrosomes is connected to the CAB in the posterior cortex. Then, in accordance with shortening of the microtubule bundle, the interphase nucleus shifts posteriorly and approaches the CAB. Consequently, an asymmetrically located mitotic apparatus is formed, and unequal division occurs. The CAB is known to be a multifunctional structure in ascidian embryos, and unequal division is not its only role (reviewed in Nishida et al. 1999; Nishida 2002). The CAB also serves as a mRNA localization scaffold. Some maternal mRNAs are specifically localized to the CAB. The CAB also contains an electron-dense matrix similar to germplasm (Iseto and Nishida 1999; Shirae-Kurabayashi et al. 2006). It is interesting that Delsman (1910) described a refractive dot within the tiny cell, probably in embryos that had been fixed and stored in glycerol. It is plausible to suggest that this was the CAB in *O. dioica*. As we did not observe the presence of the CAB in *O. dioica*, confirmation of the presence of this important embryonic structure in *O. dioica* will be a subject of future analysis.

Gastrulation

Gastrulation was initiated at the 32-cell stage, 1 h 50 min after fertilization at 13°C. This is rather earlier than in ascidians (at the 110-cell stage, for example, 9 h after fertilization at 13°C in *Halocynthia*) and other vertebrates. Internalization of the vegetal cells proceeded by ingression, keeping the embryonic outline almost spherical. There is no archenteron. Mechanisms of ingression of endodermal precursor cells have been studied in *C. elegans* embryos (Lee et al. 2006). The ingressing E blastomeres enrich myosin II apically. Their apical surfaces contract by means of contraction of actomyosin networks and this is the driving force of gastrulation. The ingression movement during gastrulation looks similar in *O. dioica* and *C. elegans*, and it is plausible that contraction of the apical surface of ingressing cells also operate in *O. dioica*.

In *O. dioica*, all vegetal cells, namely A- and B-line cells, were internalized. The gastrulation process can be subdivided into three phases. First, eight cells at the vegetal pole ingressed, and then descendants of B1 blastomeres in the posterior region followed. Finally, b22 descendants

were presumably internalized after the end of the observation period, at the 64-cell stage (T. Stach, Freie Universität Berlin, personal communication).

Neurulation

After the first phase of gastrulation, neurulation starts at the 64-cell stage. Again, neurulation begins rather earlier than in other chordates. It is difficult to precisely define the neurula stage, as the later phases of gastrulation take place simultaneously with neurulation. In the anterior region, 2×4 cells that originate from the A1 blastomere pair were internalized as in *Oikopleura longicauda* (Nishino and Satoh 2001). There was no neural fold, and folding of the neural plate was not observed. However, at the tail bud stage, four cells constituted a neural tube in optical transverse sections of nerve cord in the tail (Fig. 1m,o,q), as observed in ascidian tadpole larvae. Therefore, neural tube formation would take place at later stage after neural precursor cells have completely ingressed, although we do not know exactly when this will happen. In the present study, we did not observe internalization of the animal hemisphere cells by the 64-cell stage. However, recent cell lineage analysis showed that descendants of the a222 cell (Fig. 6b,c) join the central nervous system (T. Stach, Freie Universität Berlin, personal communication). Therefore, additional internalization will occur in the posterior region of the central nervous system (CNS) precursors. In ascidian embryos, the anterior part of the neural tube that develops into the brain is derived from a-line cells and the dorsal part of the posterior neural tube originates from b-line cells. Cells at the corresponding region in *O. dioica* do not seem to contribute to the CNS.

In *O. dioica*, embryogenesis is a rapid process and its entire life cycle lasts just 5 days. The initial morphogenetic movements such as gastrulation and neurulation take place quite early when the embryo still consists of only a small number of cells. These unique features of *O. dioica*–embryogenesis, which is extremely accelerated but conserves all the events occurring in chordate early embryos, would provide several advantages for studying the mechanisms of development of this animal using molecular and genetic approaches, and would make it an ideal representative model organism in the phylum Chordata.

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