1. Complete title of paper

Cleavage pattern and development of isolated D blastomeres in bivalves

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4. Total number of text and figures

One manuscript file, two tables, six figure files and two movie files.

5. Abbreviated title

Cleavage and development of isolated blastomeres

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7. Supporting grant information

Grant-in-Aid for JSPS Fellows. Grant number: 12J01644

Abstract

Although bivalves develop through spiral cleavage patterns, similar to other lophotrochozoans, the cleavage pattern of D lineage blastomeres is unique, since 2d shows four rounds of stereotypic unequal cleavage before bilateral cleavage of the largest derivative of 2d: $2d^{1121}$. This unique modification of spiral cleavage is directly associated with the characteristic morphology of bivalves, namely, bilaterally separated shell plates, because the bilateral shell plates are thought to be derived from the bilateral derivatives of $2d^{1121}$. In this report, to determine whether the unique cleavage pattern of bivalves is regulated depending on the interaction with other cells or by cell autonomous mechanisms, we performed cell isolation experiments and observed subsequent cleavage patterns of isolated blastomeres. When focusing on the largest derivatives of D blastomeres, 8% of isolated D blastomeres followed the cleavage pattern of normal development up to bilateral cleavage. Importantly, the remainder of the partial embryos completed cleavage before that stage, and none of the isolated blastomeres showed abnormal cleavage patterns. We also examined the development of isolated blastomeres and found that isolated D blastomeres could develop shell plates, whereas larvae developed from AB blastomeres never had shell plates. Based on these observations, we concluded that D blastomeres control their unique cleavage pattern through intrinsic mechanisms and develop shell glands autonomously without any cell-cell interaction with other lineages.

Introduction

Mollusca are one of the most divergent animal phyla characterized by calcified shells. Based on the morphology of shell plate(s), molluscs are grouped into eight classes. Recent genome-wide phylogenetic studies indicated that these eight classes are categorized into two higher groups; Aculifera and Conchifera (Kocot et al., 2011, Smith et al., 2011). Well-known taxa are included in the latter group, including Monoplacophora, Cephalopoda, Gastropoda, Scaphopoda and Bivalvia, while Chaetodermomorpha, Neomeniomorpha and Polyplacophora are classified into the former group. Common ancestors of the Conchifera are generally believed to possess a single shell on the dorsal side (Waller, '98).

During molluscan development, morphogenesis of the shell plate(s) initiates as early as the gastrula stage. Therefore, varieties in shell morphology in molluscs were likely achieved through modification during early embryogenesis.

Early embryogenesis of molluscs was first described more than 100 years ago (Lillie, 1895, Conklin, 1897, Meisenheimer, '01). These studies indicated that molluscs develop through spiral cleavage patterns, which is also observed in other animal groups such as annelids. During spiral cleavage, the first two cleavages generate four blastomeres designated as A, B, C and D, which usually correspond to the left, ventral, right, and dorsal side of the larvae stage (Lillie, 1895, Render, '91, Dictus and Damen, '97, Hejnol et al., 2007). After the 4-cell stage, each macromere buds off a small micromere at its animal side. Each quartet of micromeres is displaced to the right or left of its sister macromere. After generating the first quartet, the macromeres continue to divide unequally to generate animal micromere quartets. Thus, the largest cell in a cleavage stage is usually one of the macromeres located on the most vegetal side. This type of orthodox spiral cleavage is observed in gastropods, although some

species possess larger D lineage blastomeres due to asymmetric cell divisions, sometimes accompanied by the formation of the polar lobe. In either case, embryos develop into trochophore larvae with shell plates on the dorsal side.

Among Conchifera, bivalves acquired a novel body plan from their univalved ancestors via bilaterally separating the dorsal shell plate into two plates (Waller, '98). We investigated modifications during early development, which lead to the unique shell morphology of bivalves. The earliest sign of modification is observed as early as the spiral cleavage stage.

In bivalves, most species show unequal cleavage during their first two cleavages and give rise to a larger D cell. This unequal cleavage is not unique to bivalves but is also observed in some species of gastropods or annelids (Nielsen, 2004). The first unique modification is a reversal in polarity in the cleavage of the second D lineage micromere (cleavage of 2d and 2D). During orthodox spiral cleavage, because the vegetal blastomere is usually larger, 2D is expected to be larger than 2d. However, for bivalves, 2d is larger than 2D, and the 2d blastomere derives a bivalve shell anlage (Lillie, 1895). According to the importance of the 2d cell and its descendants in bivalve development, the 2d cell and its largest descendant are denoted as X until bilateral division (Lillie, 1895). The 2d blastomere subsequently undergoes four unequal cleavages (Lillie, 1895, Meisenheimer, '01, Guerrier, '70, Kin et al., 2009, Kurita et al., 2009). The micromeres generated from X blastomeres are labeled X^1, X^2, X^3 and X^4 in order of their generation (Figure 1A). The first two rounds of spiral cleavage are also unique and give rise to two small blastomeres on the vegetal side (X^1:$2d^2$ and X^2:$2d^{12}$). In the subsequent cleavage events, the polarity of X ($2d^{11}$) is reversed and gives rise to a small blastomere on the animal side (X^3: $2d^{111}$) and a large blastomere (X:

$2d^{112}$) on the vegetal side. During the next cleavage, the cell size polarity is reversed again for the X blastomere ($2d^{112}$) and yields a small blastomere on the vegetal side (X^4: $2d^{1122}$). After these four unequal cleavages, the largest descendant of 2d (X; $2d^{1121}$) shows bilaterally symmetric cleavage. Interestingly, the bilateral daughter cells of X, $2d^{1121}$, were described as anlages of bilateral shell glands in bivalves (Lillie, 1895). Recently, it was suggested that some descendants of the 1d blastomere and X^1 differentiate into ligament cells (Kin, et al., 2009). Therefore, the unique cleavage pattern of the bivalves is associated closely with evolution of the bivalve body plan. Here, we explored how the unique cleavage is regulated and whether the cleavage pattern is regulated by an autonomous mechanism in D blastomere or is dependent on the interaction with other blastomeres. To accomplish this, we performed cell isolation experiments.

The development of isolated blastomeres has been studied extensively in gastropods since the first study was performed more than 100 years ago (Crampton and Wilson, 1896). These studies indicated that three or four rounds of spiral cleavage occur autonomously (Crampton and Wilson, 1896, Hess, '56), and when isolated at the four-cell stage, D blastomere can develop shell glands (Cather, '67). In addition, these studies provided evidence that shell glands differentiate from any animal blastomeres of the ectoderm through induction from vegetal blastomeres (Cather, '67). However, in the bivalve *Mytilus*, Rattenbury and Berg ('54) reported that isolated D blastomere (or any other isolated blastomere) did not differentiate into shell glands (Rattenbury and Berg, '54). Here, we reexamined this observation in another species of mussel, *Septifer virgatus*, using molecular markers for shell gland differentiation.

Materials and Methods

Animals and in vitro fertilization

Sexually mature individuals of *Septifer virgatus* were collected on the Hiraiso coast, Ibaraki Prefecture, Japan, during the breeding season (July-September). *In vitro* fertilization was performed following the methods described by Kurita et al. (2009). Embryos were cultured in artificial sea water (ASW) at 25˚C.

Cell isolation

To dissolve an egg membrane, sperm extracts were prepared as described previously (Berg, '50). The two- or four-cell stage embryos were treated with sperm extract for 30 sec. After washing twice in ASW, embryos were separated by hand using a glass needle. Isolated blastomeres were collected in petri dishes. The cleavage pattern of isolated blastomeres was observed using a glass bottom dish (MATSUNAMI GLASS IND.). Embryos were cultured in ASW until fixation at 24 h post-fertilization (hpf).

Immunohistochemistry

The larvae were fixed with 4% paraformaldehyde, 0.1 M MOPS (pH 7.5), 2 mM EGTA, and 0.5 M NaCl, and stored in 100% methanol at −20°C. After rehydration, the embryos were incubated in PBT with 3% BSA for 1 h. The specimens were incubated with 1/200 mouse anti-beta-tubulin antibody (Sigma, St. Louis, MO, USA) in PBT for 1h. After rinsing four times in PBT, the samples were incubated with secondary antibody with Alexa 555 (Molecular Probes, Eugene, OR, USA) diluted 1/200 in PBT for 1 h. The samples were

washed four times in PBS and mounted on slides in 50% glycerol in PBS for observation.

In situ hybridization

In situ hybridization was performed as described previously (Kin, et al., 2009, Hashimoto et al., 2012). *Chitin synthase 1* (*CS1*) was cloned by RT-PCR using the following primer set; F: 5'-ATGAAAAGTGACATTCAGATTGGCAG-3', R: 5'-TCTGGTTCCCATCCAGGAGGAACAACATC-3'. The GenBank/EMBL/DDBJ accession number of *CS1* is AB613818.

Results

Development of isolated AB and CD blastomeres

We first performed blastomere isolation at the 2-cell stage. For cell isolation, the egg membrane was dissolved using sperm extract treatment to reduce cell adhesion (Figure 1B, C). The total numbers of isolated blastomeres were 503 for AB cells and 550 for CD cells. We observed cleavage pattern of a part of them under living conditions. A total of 87% (61/70 = cell cleavage events / cells observed) of the isolated AB cells divided into daughter cells of nearly the same size, while 90% (87/97) of CD cells divided unequally after forming the polar lobe (Figure 2, Movie.1). The remainder of the isolated blastomeres did not cleave, and no blastomeres showed abnormal cleavage patterns. These cleavage patterns of isolated blastomeres were similar to that of gastropods (Crampton and Wilson, 1896, Hess, '56).

To examine further development, we fixed surviving larvae swimming by cilia at 24

hpf. The survival rate of embryos that developed from each blastomere of the 2-cell stage was 72% (361/503 = number of surviving larvae / number of isolated blastomeres) for AB and 73% (404/550) for CD. At this stage of normal development, larvae formed D-shape morphologies with separated shell plates covering a wide part of the body (Figure 3A, B). Foot development was observed on the ventral side. The isolated AB blastomeres developed into trochophore-like larvae with prototroch, although we did not observe other structures such as the mouth, shell field, foot or apical tuft (Figure 3C, D, Table 1). Larvae from CD blastomere also developed into trochophore-like larvae with prototroch. The majority of larvae had shell plates on the dorsal side (Figure 3E, F, Table 1), but no foot-like structure was observed.

Cleavage pattern of isolated D blastomeres

To isolate D blastomeres, we treated 4-cell stage embryos with sperm extracts and isolated the largest blastomere (Figure 1.D, E). We could isolate 517 D blastomeres, and 174 cells of them were observed under living condition. Table 2 summarized the cleavage pattern of isolated D blastomeres. Among 174 isolated D blastomeres, 138 underwent two rounds of unequal cleavage (Figure 4A). We considered the largest blastomere to be 2d (X). Although in some partial embryos, 2D and 1d were located on the same side as the large blastomere 2d, this was not indicative of a reversal of unequal cleavage (in spiral cleavage, blastomeres were located in the order 1d-2d-2D from the animal pole). Because the cleavage plane is oblique and the direction of obliqueness reversed during successive cleavage, 1d and 2D are quite close during normal development (see Figure 2B, H of Kurita et al. 2009 as well as Figure 3 of Kin et al. 2009). A total of 14 embryos ended cleavage after one round of unequal

cleavage, and 22/174 never underwent cleavage.

We further monitored the development of 138 embryos by focusing only on the subsequent cleavage pattern of 2d (X) (Figure 4, Movie 2). Among 138 embryos, 22 did not show any further cell division, and 26 stopped after one round of unequal cleavage. A total of 90 embryos showed two further rounds of unequal cleavage and two small blastomeres located adjacent to 2D (Figure 4B, C). This cell arrangement is similar to that in normal development, in which $2d^2$ (X^1) and $2d^{12}$ (X^2) are located on the vegetal side of $2d^{11}$ (X). A large number of isolated blastomeres ended cleavage at this stage, and only 40 embryos proceeded to the next round of unequal cleavage. During the next cleavage, the smaller blastomere emerged at the other side of the previous small blastomeres, perhaps reflecting the reversal of polarity during normal development, in which $2d^{11}$ divided unequally into small $2d^{111}$ (X^3) and larger $2d^{112}$ (X) (Figure 4E). A total of 17 blastomeres proceeded to the next round of unequal cleavage and gave rise to smaller blastomeres on the opposite side of the previous small blastomere. This also likely reflects the cleavage of normal development, where $2d^{112}$ (X) divided unequally into larger $2d^{1121}$ (X) and smaller $2d^{1122}$ (X^4). After the above four rounds of unequal cleavages, 14 partial embryos showed a symmetric cell division, perhaps reflecting bilateral cleavage of $2d^{1121}$ (X) into $2d^{11212}$ (X^R) and $2d^{11212}$ (X^L) (Figure 4F). It should be noted that when focusing on the cleavage of X lineage, we did not observe blastomeres that underwent abnormal cleavage patterns.

Further development of isolated D-blastomeres and expression of CS1

The majority of isolated blastomeres stopped cell division of the X lineage midstream (only 14/138 proceeded to symmetric cell division of $2d^{1121}$ (X)), but cell division did proceed in

other cell lineages. Therefore, a significant number of isolated D-blastomeres could develop into swimming larvae. Indeed, 47% (241/517) of isolated D blastomeres survived and were swimming via cilia at 24 hpf. The morphology of larvae derived from D blastomeres was similar to that from CD (Figure 3E-H). We observed an apical tuft, ciliary loop prototroch and shell plate (Table 1). While ligaments were clearly observed in un-operated larvae at this stage, we could not identify ligaments in larvae from isolated D.

To further investigate shell development in larvae derived from D blastomeres, we examined the expression of *chitin synthase 1* (*CS1*). In the pacific oyster, *chitin synthase* is expressed in the mantle during the adult stage, and expression started in the trochophore larvae stage (Zhang et al., 2012). During limpet development, *chitin synthase* is a good marker for the mantle edge (Hashimoto, et al., 2012). During normal development of *S. virgatus*, the first weak signal of *CS1* was detected in the invaginated cell of the shell anlage at the early trochophore stage (Figure 5A-C). After the shell field evaginated (14 hpf), *CS1* expression was detected at the mantle edge, and a new strong signal was observed in ligament (Figure 5D-F). At 16 hpf, *CS1* expression continued in the ligament as well as at the mantle edge (Figure 5G-I). In 24 hpf larvae, strong *CS1* expression was observed at the mantle edge, while expression decreased in the ligament (Figure 5J-L).

In larvae derived from AB blastomeres, morphology was similar to that of early trochophores with prototroch, but we did not observe any sign of shell field invagination or mouth opening as described before (Figure 3C, D). No samples showed *CS1* expression (0/32 = *CS1*-expressing larvae / analyzed larvae; Figure 6A, B). On the other hand, of the larvae from D blastomeres, approximately half (53/114) had *CS1*-positive cells on the dorsal side at 24 hpf (Figure 6C-H). The majority of partial larvae derived from D blastomere failed to

evaginate shell field cells. A total of 29/114 samples showed *CS1* expression in the invaginated cells (Figure 6C, D), but 19/114 showed *CS1* signal outside of the invagination (Figure 6E, F). Only five partial larvae showed evaginated shell plates, and the *CS1* signal was observed underlying the shell matrix (Figure 6G, H). These observations indicated that shell field differentiation did not require an interaction with derivatives of A, B or C blastomeres.

Discussion

Shell field differentiation in isolated D blastomeres

In gastropods, blastomere isolation was performed extensively in the early 20th century to investigate mosaicism during early embryogenesis (Crampton and Wilson, 1896, Wilson, '04, Clement, '62, Cather, '67, Clement, '67, Verdonk and Cather, '73). In these experiments, isolated D blastomeres could always form differentiated external shell. Some authors described that even other blastomeres (A, B or C), when isolated, developed an internal shell matrix (Verdonk and Cather, '73, Cather et al., '76). However, the “internal shell matrix” was later shown that the matrix was not necessarily indicative of differentiation of the shell matrix, but it may reflect abnormal specification of statocysts (McCain, '92). We found that isolated D blastomeres can differentiate shell glands in bivalve. However, this fact does not necessarily indicate that the shell gland can differentiate autonomously without any cell-cell interaction. Indeed, Cather ('67) indicated that, while isolated D blastomeres reached the veliger larvae stage with shell plates in *Ilyanassa*, depletion of vegetal blastomeres such as 2D or 3D resulted in loss of the shell plate. He also performed further experiments and demonstrated an inductive role of vegetal blastomeres for the differentiation of shell glands.

Blastomeres on the animal side when isolated at the 32-cell stage develop into hollow ball-like structure and do not differentiate into a shell gland. However, these animal blastomeres develop external shell matrices when combined with either macromere (3Q), mesentoblast, or even with isolated polar lobe. This induction of shell glands may account for the discrepancy in cell lineages between species. In most species, the shell glands originate from 2d blastomere, and the shell glands are derived from different lineages in some species, such as *Patella vulgata* (Dictus and Damen, '97). Despite extensive studies in gastropods, blastomere isolation has rarely been performed, and in a study using the mussel species *Mytilus edulis*, isolated D blastomeres did not develop shell plates (Rattenbury and Berg, '54). In the present study, using another species of mussel, *Septifer virgatus*, we found that larvae from isolated D blastomeres express the shell gland marker *CS1* (Figure 6) and secrete shell matrices (Figure 3, Table 1). This difference in isolated D blastomeres from different mussel species to develop shell plates may reflect different regulatory mechanisms between the two species, or it may be due to damage during experiments. Future research will determine whether induction from vegetal blastomeres is also required for shell gland differentiation in bivalves.

Unique cleavage pattern of X blastomere is controlled autonomously

Bilateral separation of shell plates is a characteristic feature of bivalves. Previous studies indicated that the unique regulation of symmetric cell division following asymmetric cleavages of 2d lineage cells is closely associated with the unique morphology of shell plates (Lillie, 1895, Kin, et al., 2009). In this study, we explored whether the unique cleavage pattern of 2d lineage is regulated autonomously or through interactions with other cells. We

found that, even though a number of isolated D blastomeres end cleavage before bilateral cleavage, approximately 8% of isolated blastomeres followed the normal cleavage pattern up until bilateral cell division (Table 2). Notably we did not observe any isolated blastomeres showing abnormal cleavage patterns, i.e., isolated blastomeres either ended cleavage or followed the normal cleavage pattern. Based on these observations, we concluded that the unique cleavage pattern did not require an interaction with cells originating from A, B or C blastomeres. Rather, the unique cleavage pattern is controlled by intrinsic mechanisms within the D lineage.

The regulatory mechanisms for asymmetric cell division is well-studied in model organisms, such as *Drosophila*, yeast and *C. elegans*, as well as in vertebrate cells (Betschinger and Knoblich, 2004). These studies revealed a conserved regulatory mechanism for asymmetric cell division, where aPKC and Par proteins are localized in the specific site of the cell membrane and regulate asymmetric cell division by attracting centrosomes (Gonczy, 2008). These molecules are also thought to be involved in the regulation of asymmetric cleavage of early development in marine invertebrates such as ascidians and sea urchins (Patalano et al., 2006, Alford et al., 2009). Although involvement of these factors in the regulation of the unique cleavage of bivalves will be explored in our future studies, the regulation of asymmetric cell division in bivalves may be more complicated than in sea urchin or ascidians. For ascidians and sea urchins, polarity of asymmetric cell division does not change during development; centrosomes are always pulled toward the vegetal pole in sea urchins (Dan, '79) and towards the posterior pole in ascidians (Hibino et al., '98). However, for 2d blastomere of bivalves, after giving rise to two micromeres on the vegetal side ($2d^2$ -X^1 and $2d^{12}$ -X^2), reversal of asymmetry occurs and produces micromeres on the animal side

($2d^{111}$-X^3). Subsequently, the reversal occurs again and produces a micromere on the vegetal side ($2d^{1122}$-X^4), prior to symmetric bilateral cleavage. We demonstrated that these reversals in asymmetry occur without interaction with other lineage cells (A, B or C lineages). Although it is possible that the cell interaction with 2D lineage or 1d lineage cells regulate the reversal of asymmetry, our results suggested that D lineage blastomeres possess intrinsic mechanisms for counting cell division. Similar counting mechanisms were proposed for *Xenopus* mid-blastula transition (MBT) or ascidian muscle differentiation. For the *Xenopus* MBT, the ratio between cytoplasmic vs. nuclear volumes is thought to be an important counting mechanism for initiating zygotic transcription in the mid-blastula stage (Newport and Kirschner, '82). Ascidians were proposed to possess distinct counting mechanisms; they regulate the commitment to muscle differentiation based on the number of DNA replication cycles (Satoh and Ikegami, '81). The reversal of asymmetric cell division can be used as a system to study the regulation of developmental timing. Furthermore, establishment of this unique mechanism may have been essential for evolution of the bivalve body plan.

Acknowledgments

NH is supported by JSPS pre-doctoral research fellowship.

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Tables

Table 1. Effect of cell isolation on the larval morphology at 24 hpf.

Table 2. The successful number of X lineage cleavage.

Figure legends

Figure 1. Cleavage pattern of X (2d) blastomere and effect of sperm extracts. (A) Summary of cleavage pattern of X (2d). Arrows indicate the cleavage order. Modified after Kurita et al. (2009). (B, C) 2-cell stage embryo before (B) and after sperm extract treatment (C). (D, E) 4-cell stage embryo before (D) and after sperm extract treatment (E). Scale bars: 50 μm.

Figure 2. Cleavage patterns of blastomeres isolated at the 2-cell stage after sperm extract treatment. Upper and lower panels show the time course of cleavage of blastomeres isolated from a single fertilized egg (upper and lower panel show similar stages after fertilization). (A) Isolated CD blastomeres showed unequal cleavage with polar lobes (arrow head). (B) Isolated AB blastomeres divided symmetrically. Movie 1 corresponds to these figures. Scale bars: 50 μm.

Figure 3. Morphology of larvae developed from isolated blastomeres at 24 hpf. (A, B) Larvae showing normal development. (C, D) Larvae developed from isolated AB blastomeres. (E, F) Larvae developed from isolated CD blastomeres. (G, H) Larvae developed from isolated D blastomeres. (A, C, E, G) Light image of the larvae. (B, D, F, H) Ciliary structures visualized using anti-beta-tubulin antibody of the larvae (ventral to the left). Arrow head: apical tuft, f: foot, dashed line: mantle edge. Scale bars: 50 μm.

Figure 4. Cleavage pattern of isolated D blastomere after sperm extract treatment. (A) Isolated D blastomeres after 2 rounds of cell divisions. (B, C) X (2d) divided unequally and

smaller X^1 ($2d^2$) (B) and X^2 ($2d^{12}$) (C) localized on the same side as 2D. (D) 2D and $1d^2$ blastomeres divided, and the third division of X ($2d^{11}$) started. (E) X^3 ($2d^{111}$) was localized on the opposite side of X^1 ($2d^2$) and X^2 ($2d^{12}$). (F) After X^4 ($2d^{1122}$) divided on the opposite side of X^3 ($2d^{111}$), X ($2d^{1121}$) divided bilaterally. The dashed line shows the lineage of X (2d) blastomeres. Movie 2 corresponds to these figures. Scale bars: 50 μm.

Figure 5. Expression patterns of *CS1* during normal development. (A-C) 10 hpf larvae. (D-F) 14 hpf larvae. (G-I) 16 hpf larvae. (J-L) 24 hpf larvae. (A, D, G, J) Dorsal view of larvae. (B, E, H, K) Lateral view and focus on the mid line of larvae. (C, F, I, L) Lateral view and focus on the right surface of larvae. Arrow head: invagination site. Dashed line: shell field. Scale bars: 50 μm.

Figure 6. Expression patterns of *CS1* in larvae developed from isolated blastomeres. (A, B) Larva developed from AB blastomeres. (C-H) Larva developed from D blastomeres. (A, C, E, G) Dorsal view. (B, D, F, H) Lateral view. Dashed line shows the shell field edge. Scale bars: 50 μm.

Movie 1. Cleavage pattern of blastomeres isolated at the 2-cell stage from a single fertilized egg after sperm extract treatment. Left is CD blastomere and right one is AB.

Movie 2. Cleavage pattern of isolated D blastomere after sperm extract treatment. The dashed line shows the lineage of X blastomeres.

Tables

Table 1. Effect of cell isolation on the larval morphology at 24 hpf.

Isolated blastomere	# of samples	ciliary loop	apical tuft	shell plates	mouth
AB	19	19	0	0	0
CD	20	20	15	13	12
D	26	26	15	17	16

Table 2. The successful number of X lineage cleavage.

Total number	1d-1D	X (2d)	X^1 ($2d^2$)	X^2 ($2d^{12}$)	X^3 ($2d^{111}$)	X^4 ($2d^{1122}$)	X^L&X^R ($2d^{11211}$ & $2d^{11212}$)
174	152	138	116	90	40	17	14
%	87.3	79.3	66.7	51.7	23	9.8	8

Figures

Fig.1

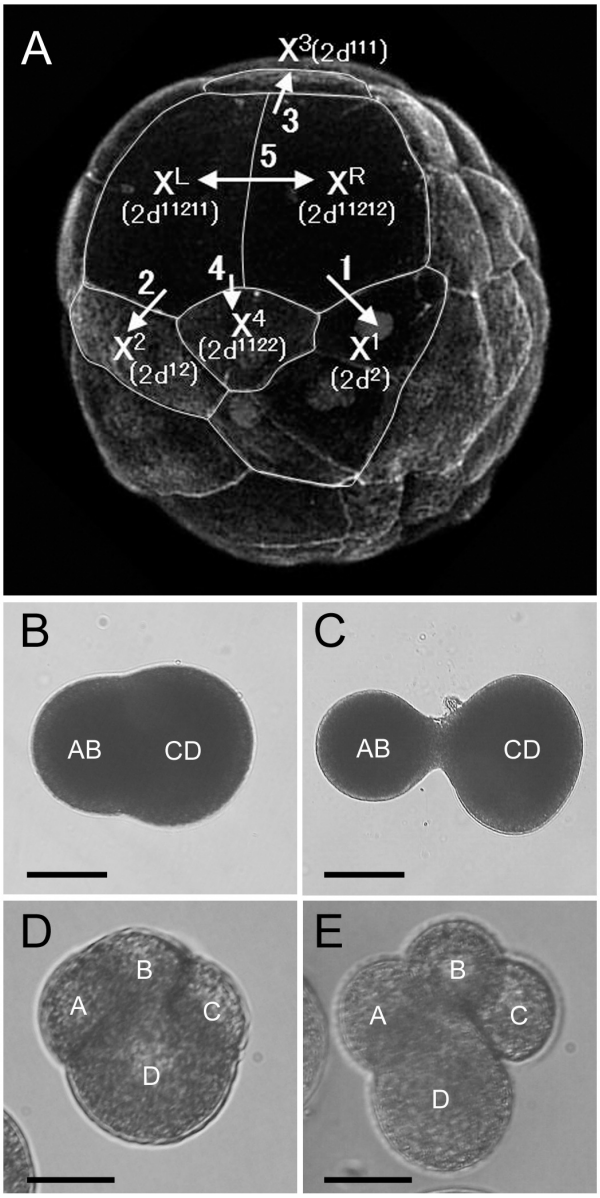

Fig. 2

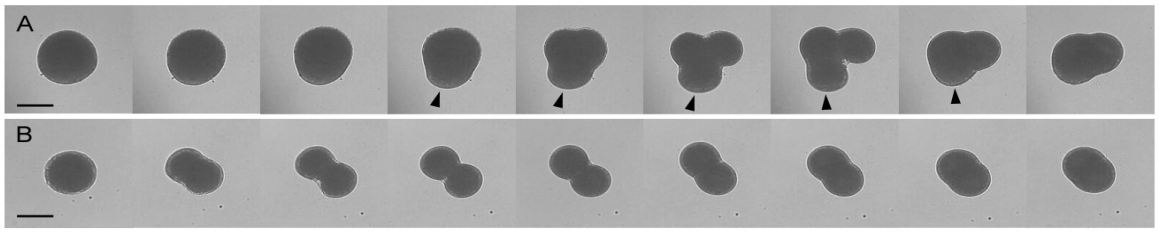

Fig.3

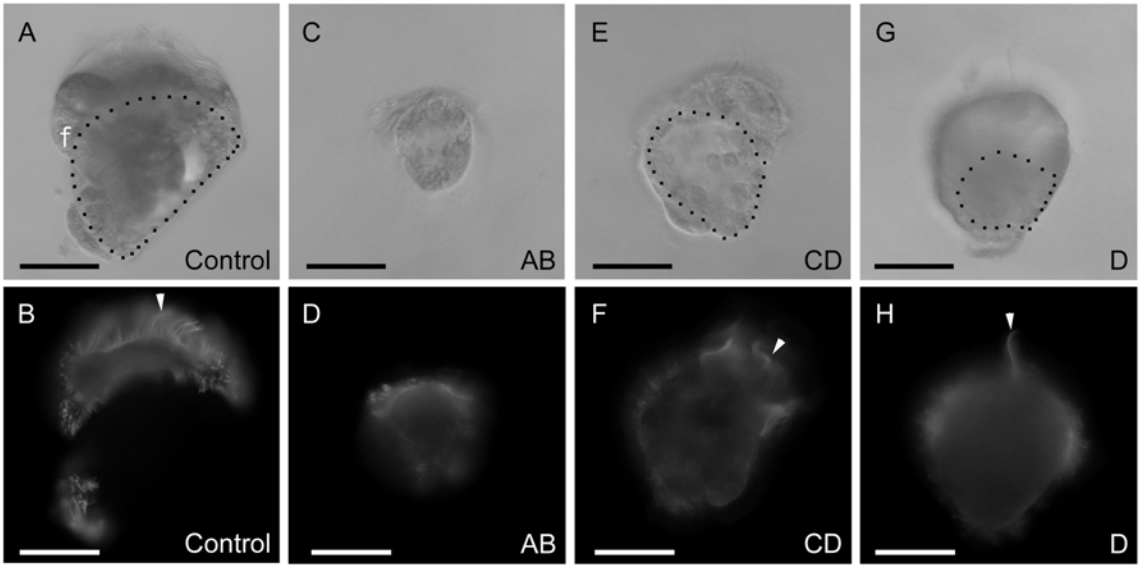

Fig.4

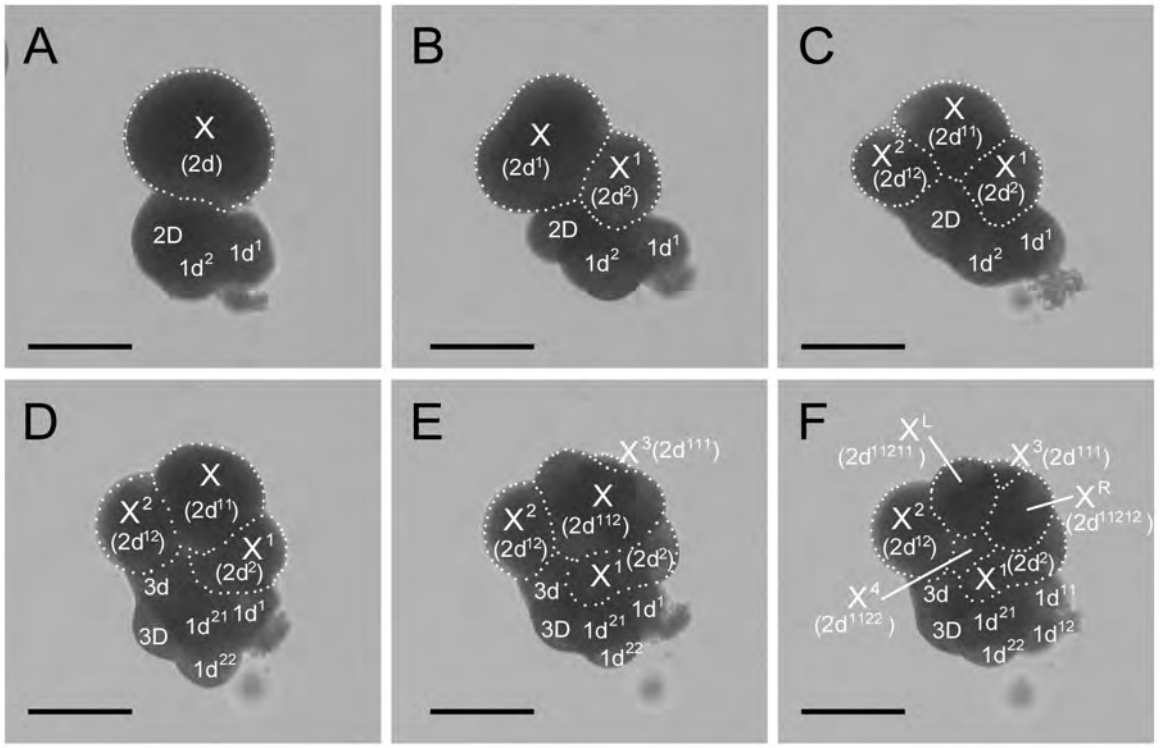
A
X
(2d)
2D
1d2
1d1
B
X
(2d1)
X1
(2d2)
2D
1d2
1d1
C
X
(2d11)
X2
(2d12)
X1
(2d2)
2D
1d2
1d1
D
X
(2d11)
X2
(2d12)
X1
(2d2)
3d
3D
1d21
1d1
1d22
E
X3(2d111)
X
(2d112)
X2
(2d12)
X1(2d2)
3d
3D
1d21
1d1
1d22
F
XL
(2d11211)
X3(2d111)
XR
(2d11212)
X2
(2d12)
X1(2d2)
X4
(2d1122)
3d
3D
1d21
1d11
1d12
1d22

Fig.5

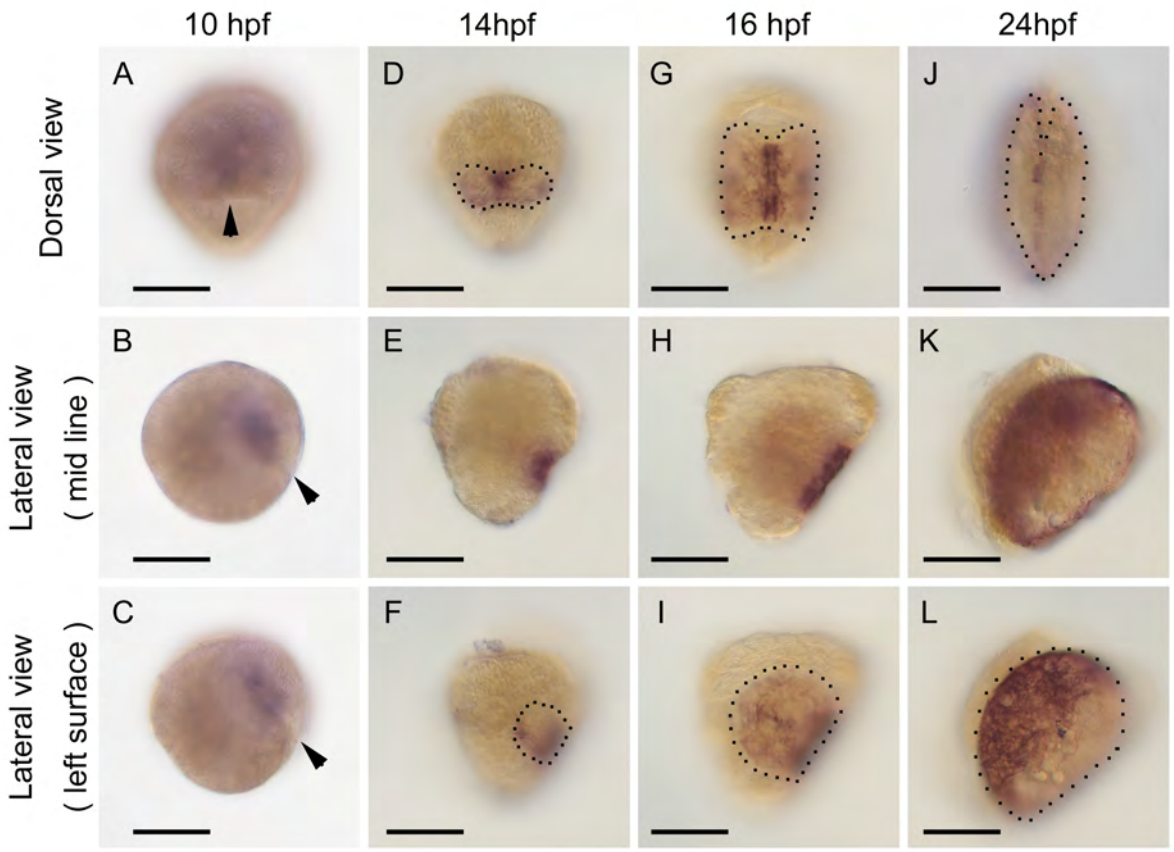
10 hpf
14hpf
16 hpf
24hpf
Dorsal view
Lateral view (mid line)
Lateral view (left surface)
A
B
C
D
E
F
G
H
I
J
K
L

Fig.6