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Behavior of Pigment Cells Closely Correlates the Manner of Gastrulation in Sea Urchin Embryos

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ABSTRACT—To know whether behavior of pigment cells correlates the process of gastrulation or not, gastrulating embryos of several species of regular echinoids (*Anthocidaris crassispina*, *Mespilia globulus* and *Toxopneustes pileolus*) and irregular echinoids (*Clypeaster japonicus* and *Astriclypeus manni*) were examined. In *M. globulus* and *A. crassispina*, the archenteron elongated stepwise like in well-known sea urchins. In the embryos of both species, fluorescent pigment cells left the archenteron tip and migrated into the blastocoel during gastrulation. In *T. pileolus*, *C. japonicus* and *A. manni*, on the other hand, the archenteron elongated at a constant rate throughout gastrulation. In these species, no pigment cell was observed at the archenteron tip during invagination processes; pigment cells began to migrate in the ectoderm from the vegetal pole side toward the apical plate without entering the blastocoel. These results clearly indicate that the behavior of pigment cells closely correlated the manner of gastrulation. Further, it was examined whether the archenteron cells are rearranged during invagination, by comparing the number of cells observed on cross sections of the archenteron at the early and late gastrula stages. The rearrangement was not conspicuous in *A. crassispina* and *M. globulus*, in which archenteron elongated stepwise. In contrast, the archenteron cells were remarkably rearranged in *C. japonicus*, alothough the archenteron elongated continuously. Thus, neither the behavior of pigment cells nor the manner of gastrulation matches the current taxonomic classification of echinoids.

Key words: cell movement, gastrulation, pigment cell, sea urchin, sand dollar

INTRODUCTION

Gastrulation is the most prominent morphogenetic event in early development. For analyzing the cellular and mechanical basis of gastrulation, sea urchin embryos have been used as a model system, owing to their clarity and simple organization. Gastrulation in sea urchin embryos can be regarded as the invagination of a mono-layered epithelium (Gustafson and Wolpert, 1967), and has been thought to proceed stepwise, i.e., two elongation phases are intervened with a lag phase in which the archenteron scarcely elongates (Gustafson and Kinnander, 1956; Dan and Okazaki, 1956). In the first elongation phase, the thickened vegetal plate bends inwardly and gives rise to a short stub-like gut rudiment (primary invagination). As has been pointed, this process is autonomous, since the isolated vegetal plate still forms a gut rudiment morphologically similar to that forms in normal embryos (Moore and Burt, 1939; Ettensohn, 1984). Bottle cells are thought to produce the mechanical force for bending of the vegetal plate (Nakajima and Burke, 1996; Kimberly and Hardin, 1998). During the lag phase, secondary mesenchyme cells (SMCs) appear at the tip of the gut rudiment, and extend thin and long filopodia toward the apical plate. It is generally thought that constriction of such filopodia pulls the gut rudiment upwardly (Dan and Okazaki, 1956; Hardin, 1988). The rearrangement of archenteron cells is another important cellular basis for the elongation of the gut rudiment (Ettensohn, 1985; Hardin and Cheng, 1986; Hardin, 1989). As a result, a slender archenteron forms by the end of the second phase of invagination (secondary invagination).

This scheme of gastrulation matches well the processes of gastrulation observed in a regular echinoid *Hemicentrotus pulcherrimus*. Before the onset of primary invagination, bottle cells are observed at the central region of the vegetal plate (Takata and Kominami, 2001). After primary invagination, SMCs appear at the archenteron tip and extend filopodia toward the apical plate. During secondary invagination, archenteron cells are stretched along the axis of the archenteron, showing the existence of a tension exerted by SMCs (Kominami and Takata, 2000). Number of cells observed in a cross section of the archenteron

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becomes smaller as invagination proceeds, indicating that the archenteron cells are rearranged during gastrulation (Kominami and Masui, 1996). Further, the invagination proceeds autonomously, i.e., independently of the force exerted by the ectodermal layer surrounding the vegetal plate (Takata and Kominami, 2001). The processes of gastrulation in *Echinometra mathaei* (regular echinoid) are almost the same as those observed in *H. pulcherrimus* (Takata and Kominami, 2004). Also in *Mespilia globulus* and *Pseudocentrotus depressus*, the archenteron looks to elongate stepwise, as far as judged from the external morphology (Okazaki, 1975).

The scheme, however, is not applicable to an irregular echinoid *Scaphechinus mirabilis*. The archenteron elongates continuously at a constant rate throughout gastrulation, hence the distinction between primary and secondary invagination cannot be noticed (Kominami and Masui, 1996; Kominami and Takata, 2000). Bottle cells are scarcely observed in the vegetal plate (Takata and Kominami, 2001). Although SMCs appear at the archenteron tip during invagination, they do not extend filopodia toward the apical plate and do not look to exert pulling force (Kominami and Takata, 2000). In fact, the constituent cells are not stretched along the axis of the archenteron. Further, it was shown that blastoderm is continuously involuted into the base of the archenteron throughout gastrulation, and that the force exerted by ectoderm is indispensable for the progress of invagination (Kominami and Takata, 2000; Takata and Kominami, 2001). Thus, the manner of gastrulation is quite different between regular and irregular echinoids so far examined.

In a previous study, we found that pigment cells become noticeable as early as the onset of gastrulation in *E. mathaei*, owing to the very early accumulation of pigment granules (Takata and Kominami, 2003). Taking advantage of this property, it was elucidated that pigment cells act as bottle cells at least in *E. mathaei* (Takata and Kominami, 2004). In the consequence of this finding, it became a problem of high priority to learn how pigment cells behave during gastrulation in a variety of echinoids. Pigment cells, however, generally become visible at the late gastrula or early prism stage. Due to this, it has long been unclear how pigment cells behave during gastrulation. A few years ago, we found that pigment cells fixed with formalin emanate autofluorescence upon ultraviolet (UV) or green light (G) irradiation (Kominami *et al*., 2001). As is well known, pigment cells in sea urchin embryos contain carotenoids and naphthoquinones (Monroy *et al.,* 1951; Griffiths, 1966; Matsuno and Tsushima, 2001). Fixation with formalin is thought to destruct carotenoids-protein complex (Lakshman and Okoh, 1993), resulting in the emission of autofluorescence under UV- or G-illumination. Although it has been known that some genes, such as *SpHmx* (Martinez and Davidson, 1997), *S9* and *CyIIa* (Miller *et al.,* 1996), are expressed specifically in pigment cells, timing of the specific expression of these genes does not differ from the timing of pigment granule accumulation. Presently, detection of the autofluorescence is the most sensitive and convenient method to observe the behavior of pigment cells. Using this method, we have already elucidated the behavior of pigment cells in some echinoids. In *H. pulcherrimus* (Kominami *et al.,* 2001), pigment cells are positioned at the archenteron tip during invagination, and leave the archenteron at the end of gastrulation. These pigment cells enter the apical plate, and then migrate toward the vegetal pole through the aboral ectoderm. In *S. mirabilis* embryos, on the other hand, pigment cells begin to disperse in the vegetal plate before the onset of gastrulation (Kominami *et al.,* 2001). Then, they migrate toward the apical plate through the ectodermal layer without entering the blastocoel. It is of note that no pigment cell is observed at the archenteron tip during gastrulation in this species. Thus, the behavior of pigment cells is also quite different between regular and irregular echinoids, as well as the manner of gastrulation.

These observations raise the possibility that the manner of gastrulation might depend on how pigment cells behave during gastrulation. Are pigment cells necessary for the stepwise elongation of the archenteron? If pigment cells are absent at the archenteron tip, does the archenteron elongate continuously? Does the difference in the manner of gastrulation or the behavior of pigment cells reflect the taxonomic position? To address these questions, we observe the manner of gastrulation and behavior of pigment cells in several species of regular and irregular echinoids in this study. Further, we examine the hybrid embryos made by inseminating *S. mirabilis* eggs with *H. pulcherrimus* sperm, to learn what degree the manner of gastrulation or the behavior of pigment cells is maternally programmed.

MATERIALS AND METHODS

Animals and gametes

Six regular echinoids (*Hemicentrotus pulcherrimus*, *Echinometra mathaei*, *Anthocidaris crassispina*, *Mespilia globulus*, *Temnopleurus toreumaticus* and *Toxopneustes pileolus*) and three irregular echinoids (*Scaphechinus mirabilis*, *Clypeaster japonicus*, and *Astriclypeus manni*) were used in the present study. Hereafter, abbreviations listed in Table 1 will be used to represent species names. Among species listed above, embryos of four regular echinoids (*Ac*, *Mg*, *Tp* and *Tt*) and two irregular echinoids (*Cj* and *Am*) were examined with respect to the manner of gastrulation and behavior of pigment cells.

Adults of *Em*, *Ac*, *Mg* and *Tp* were collected at the south district of Ehime prefecture during the breading season. Adults of *Tt* and *Cj* were kindly provided from Usa Marine Laboratory (Kochi Univ., Kochi, Japan). Adults of *Hp*, *Sm* and *Am* were collected at the seashore near Matsuyama city. Adults and embryos of *Hp*, *Em* and *Sm* were handled as described before (for *Hp* and *Sm*, Kominami and Masui, 1996; for *Em*, Takata and Kominami, 2003). Animals of other species were kept in aquaria with aeration at 24°C. Gametes were handled with a standard method, and embryos were cultured at 24°C. Millipore-filtered seawater (MFSW) was supplemented with 100 units /ml penicillin and 50 μg/ml streptomycin.

Histological observation of gastrulating embryos

Gastrulating embryos were fixed with 2% glutaraldehyde for 2 hr at a room temperature and dehydrated with a graded ethanol

Table 1. Manner of gastulation and behavior of pigment cells.

Species	Abbreviation	<Manner of gastrulation>						<Behavior of pigment cells>		
		change in			Final degree of invagination (%)	rerrangement[¶] of archenteron cell	Type of[§] elongation	Appearing[§] position	Detectable[§] from	Number of cells
		height	width	thickness						
*Hemicentrotus pulcherrimus**	*Hp*	–	++	–	84±1	++	Step	blasto	mid-to-late gas	50
*Echinometra mathaei***	*Em*	+	++	––	94±2	+	Step	blasto	early gas	30
Anthocidaris crassispina	*Ac*	±	++	–	84±6	–	Step	blasto	early gas	60
Mespilia globulus	*Mg*	±	±	–	52±6	–	Step	blasto	late gas[†]	20
Toxopneustes pileolus	*Tp*	±	+	––	94±4	+	Cont	ecto	early gas	30
Temnopleurus toreumaticus	*Tt*	±	±	±	93±4	–[‡]	Cont	–	pluteus	20
Clypeaster japonicus	*Cj*	–	++	–	92±6	++	Cont	both	early gas	30
Astericlypeus manni	*Am*	+	±	±	70±4	–	Cont	ecto	early gas	300
*Scaphechinus mirabilis**	*Sm*	–	±	±	84±4	–	Cont	ecto	early gas	80
Hybrid (*Hp* : *Sm*)	Hybrid	–	++	±	83±8	–[‡]	Cont	both	mid-gas	60–90

* Data from Kominami *et al.* (2001).
** Data from Takata and Kominami (2004).
† Autofluorescence is detected under only UV-irradiation, while pigment cells are fluorescent under both UV-and G-irradiation in other species.
‡ Juudged on photographic prints of whole embryos.
¶ Judged at the late gastrula stage when the degree of invagination reached the plateau.
§ Step: stepwise invagination. Cont: continuous invagination. blasto: blastocoel. ecto: ectoderm. gas: gastrula

series. The specimens were embedded in Spurr resin and sectioned (1 μm in thickness) with an ultramicrotome (Porter-Blum, Newtown, CT, USA). The sections were stained with 1% toluidine blue and photographed. The number of cells observed in cross-sections of the archenteron was counted on photographic prints.

Observation of pigment cells

Embryos were fixed with 10% formalin (Wako Pure Chemical, Osaka) in MFSW for several minutes, and mounted on a glass slide using two strips of vinyl tape (thickness, about 100 μm) as spacers. The specimens were examined under an epifluorescence microscope (BX50-FLA, Olympus, Tokyo), and photographed under UV- or G-illumination. For detailed observation of the distribution of pigment cells, embryos were loaded into an observation chamber (Kominami *et al.,* 2001), and examined from both dorsal and ventral sides by inverting the chamber.

Hybrid formation

Dry sperm of *Hp* was a kind gift from Tateyama Marine Laboratory (Ochanomizu Univ., Tateyama, Chiba, Japan). Eggs of *Sm* were inseminated with *Hp* sperm suspension at a higher concentration than usually employed. Ten minutes after insemination, fertilized eggs were collected under an inverted microscope using a micropipette, and cultured at 18°C. Frequency of successful fertilization was variable among batches of eggs (2–60%). Although reverse cross-fertilization was tested during the natural breading season of *Hp*, eggs of *Hp* were never fertilized with *Sm* sperm.

RESULTS

Changes in embryo morphology during gastrulation

Fig. 1 shows the gastrulating embryos of six species examined in this study. Photographs in the first column show the embryos just at the beginning of gastrulation. Photographs in the second, third and fourth column show the embryos during early, middle and late phase of gastrulation, respectively.

The blastocoel of *Ac* early gastrula was narrow and filled with primary mesenchyme cells (PMCs, Fig. 1A). The blastocoel wall was thick at early phase of gastrulation and became thinner as invagination proceeded (Fig. 1B–D). Changes in the height and width of the embryo, and change in thickness of the blastoderm during gastrulation are represented as '–– ' ~ '++' according to their degrees, and shown in Table 1. The degree was determined by examining more than 30 fixed embryos or histological specimens, and defined as '+' or '–', if the change is 5–15% increase or decrease, respectively. If the increase or decrease exceeds 15%, '–– ' or '++' is given, respectively. When the change is less than 5%, '+' is marked. The morphological changes observed in *Ac* embryos are similar to those observed in *Em* (Takata and Kominami, 2004).

In *Mg* (Fig. 1E–H) and *Tp* (Fig. 1I–L), the blastoderm had already expanded by the onset of gastrulation (Fig. 1E, I). From the early phase of invagination, a number of SMCs left the archenteron tip, and were dispersed in the blastocoel (Fig. 1G, K). In these species, changes in the height and width of embryos, and change in the thickness of the blastoderm were scarcely noticed during gastrulation (Table 1). At the end of gastrulation, a slender archenteron formed (Fig. 1H, L). It is of note that the archenteron of *Mg* embryos never reached the apical plate (Fig. 1H). The blastoderm of *Tt* embryos (Fig. 1M–P) showed a moderate thickness. A considerable number of SMCs appeared at the archenteron tip from the early phase of gastrulation. The archenteron formed at the end of invagination (Fig. 1P) was relatively larger than those in other species.

The contour of *Cj* mesenchyme blastulae around the onset of gastrulation (Fig. 1Q) resembled that of *Am* embryos, except the thickness of the blastoderm (Fig. 1U). In *Cj* embryos, however, the apical plate and the prospective region of the post-oral arms, at which two ventro-lateral clusters of PMCs formed, became angular as gastrulation proceeded (Fig. 1R–T). A chain of SMCs sometimes connected

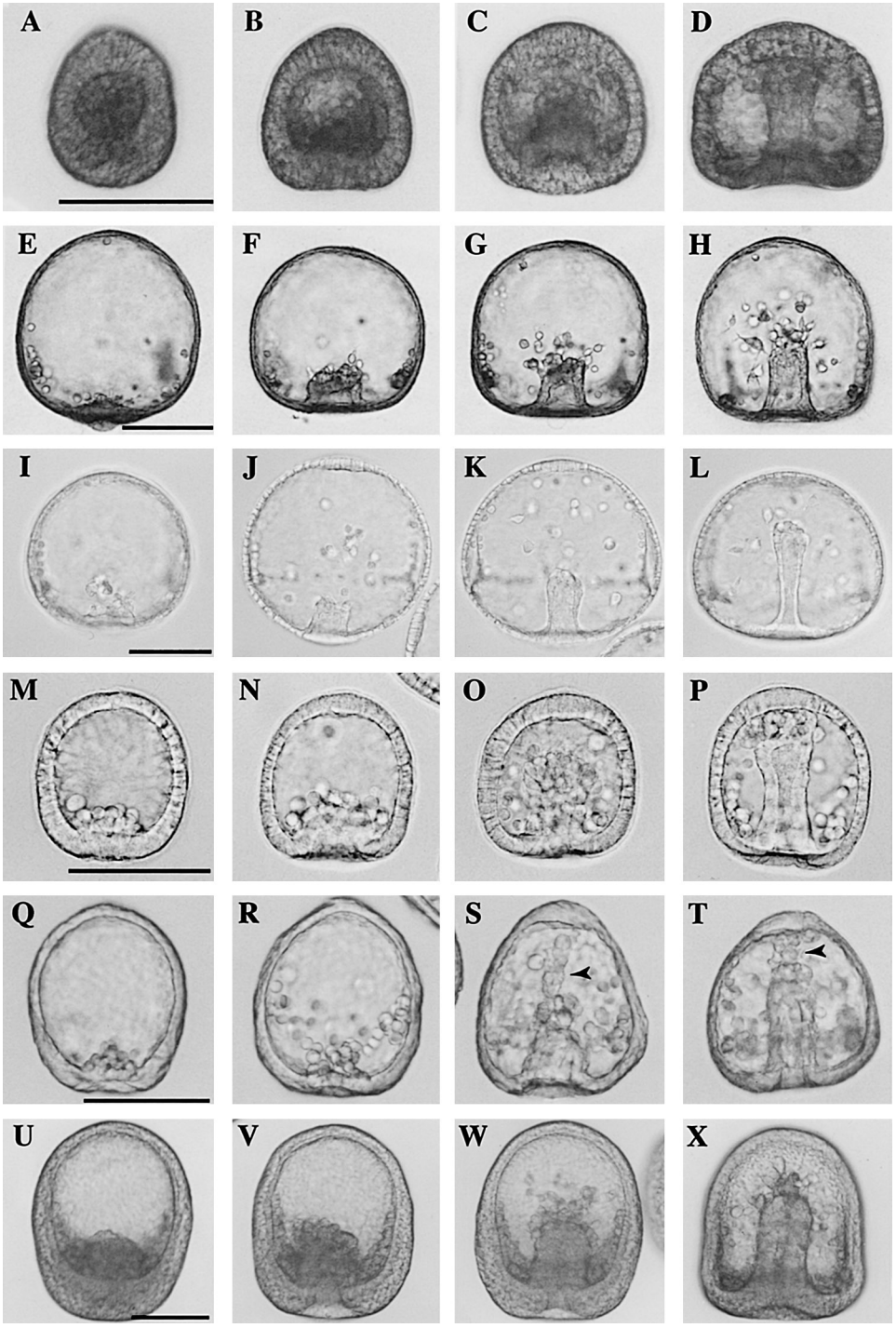

Fig. 1. *Morphology of embryos during gastrulation.* A–D: *A. crassispina*, 12, 14, 16, and 18 hours post-fertilization (hr). E–H: *M. globulus*, 10, 11, 12, and 14 hr. I–L: *T. pileolus*, 22, 24, 26, and 28 hr. M–P: *T. toreumaticus*. 14, 16, 18, and 20 hr. Q–T: *C. japonicus*, 16, 20, 24, and 28 hr. U–X: *A. manni*. 12, 14, 16, and 18 hr. The first, second, third and fourth columns show the embryos at the beginning of gastrulation, early gastrula stage, mid-gastrula stage and late gastrula stage, respectively. Note that morphologies of gastrulating embryos are quite different among species. Arrowheads in S and T indicate chains of secondary mesenchyme cells. Each scale bar is common to photographs in the same row, and indicates 100 μm.

the archenteron tip and the inner surface of the apical plate (Fig. 1S, T, arrowheads). Morphology of gastrulating *Am* embryos (Fig. 1U–X) resembled *Sm* embryos at each stage, whereas the blastocoel wall was thicker and more opaque. Like in *Mg* embryos, the archenteron did not reach the apical plate in *Am* (Fig. 1X).

Archenteron elongation

To know whether the archenteron elongates stepwise or continuously, degrees of invagination (ratios of the arch-

enteron length to the height of the blastocoel) were measured in gastrulating embryos of six species (Fig. 2). In *Ac* embryos, a short lag phase in the archenteron elongation was noticed after primary invagination (Fig. 2A, between single and double arrowheads). In *Mg* embryos, a typical stepwise pattern of archenteron elongation was observed (Fig. 2B, between single and double arrowheads), although the final degree of invagination was 50% at most. Interestingly, *Tp* and *Tt* embryos, both classified to regularia, showed a typical continuous invagination (Fig. 2C, D). Thus, it became clear that both patterns of archenteron elongation are observed among regular echinoids. In two irregular echinoids *Cj* and *Am*, invagination proceeded continuously, as shown in Figs. 2E and 2F, respectively.

Another important factor for the archenteron elongation is the occurrence of the rearrangement of archenteron cells, which can be easily known by comparing the number of cells observed on cross-sections of the archenteron at the mid-level (Fig. 3). In *Ac* embryos, almost the same numbers of cells were observed on the cross-sections obtained from early and late gastrulae (Fig. 3A, B), indicating that the rearrangement does not occur. In contrast to this, occurrence of the rearrangement of archenteron cells was clear in *Em*, *Tp* and *Cj* embryos (Fig. 3C–D, G–H, I–J, respectively). In these species, more slender archenteron formed at the end of gastrulation. In *Mg* (regularia) and *Am* (irregularia), almost the same numbers of cells were observed in cross sections of the archenteron of the early and late gastrulae (Fig. 3E–

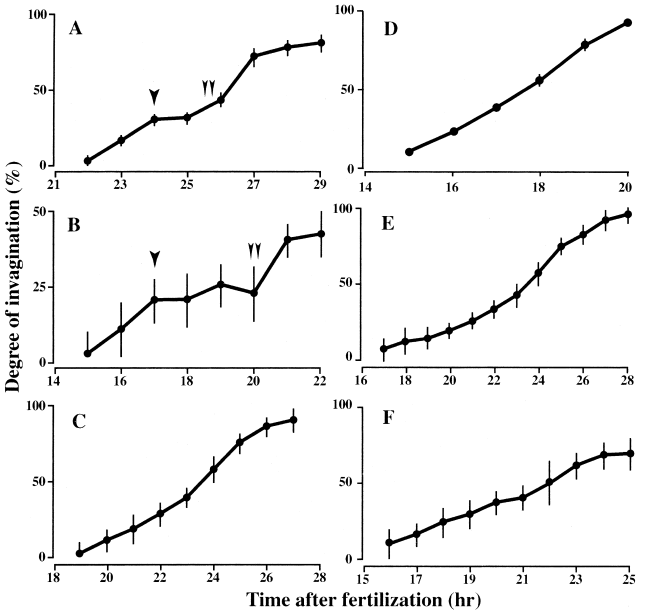

Fig. 2. *Change in the degree of invagination.* A: *A. crassispina*. B: *M. globulus*. C: *T. pileolus*. D: *T. toreumaticus*. E: *C. japonicus*. F: *A. manni*. Abscissa: time after fertilization (hr). Ordinate: degree of invagination (ratio of the archenteron length to the height of the blastocoel). Each point represents the average value of 15 embryos. Vertical lines attached to the solid circles indicate S.D. In *A. crassispina* and *M. globulus*, the archenteron elongates stepwise. Single and double arrowheads indicate the beginning of primary and secondary invagination, respectively. In *T. pileolus*, *T. toreumaticus*, *C. japonicus* and *A. manni*, the archenteron elongate continuously, hence the distinction between primary and secondary invagination cannot be noticed.

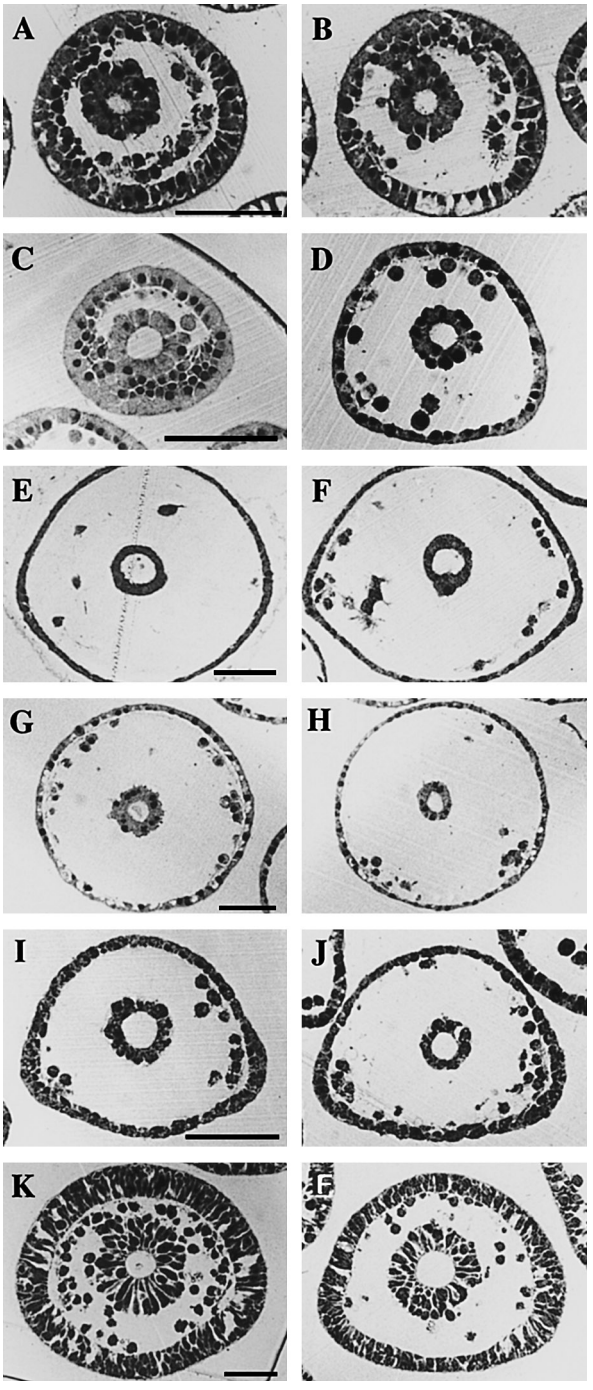

Fig. 3. *Cross section of the archenteron at the early and late gastrula stage.* A, B: *A. crassispina*. C, D: *E. mathaei*. E, F: *M. globulus*. G, H: *T. pileolus*. I, J: *C. japonicus*. K, L: *A. manni*. A, C, E, G, I and K: early gastrulae. B, D, F, H, J and L: late gastrulae. In *E. mathaei* (C, D), *T. pileolus* (G, H) and *C. japonicus* (I, J), number of cells observed on the cross section of the archenteron becomes smaller during gastrulation. In the other species, the number remains constant during the elongation of the archenteron. Each scale bar is common to photographs in the same row, and indicates 50 μm.

F and K–L, respectively). In these species, archenteron cells are not rearranged during invagination processes.

Behavior of pigment cells

Fig. 4 shows pigment cells in gastrulating embryos of *Ac* (Fig. 4A, B), *Mg* (Fig. 4C, D), *Tp* (Fig. 4E–H) and *Am* (Fig. 4I–L). In *Ac*, fluorescent pigment cells appeared at the archenteron tip when the embryos developed into the early gastrulae (Fig. 4A, B). In *Mg*, pigment cells became visible after they left the archenteron tip. At this stage, 40–50 SMCs were observed in the blastocoel, whereas about a half of them (about 20) were fluorescent (Fig. 4C, D, arrowheads). In *Tp* and *Am*, pigment cells migrated from the vegetal pole side toward the apical plate through the aboral ectoderm (Fig. 4E–H, I–L, respectively). Detailed behavior of pigment cells in *Ac* embryos is shown in Figure 5. As stated above, all pigment cells were seen in the blastocoel at the very early stage of gastrulation (Fig. 4A, B). Once gastrulation started, some pigment cells left the archenteron tip and soon entered the ectoderm near the vegetal pole (Fig. 5A, D). In a top view of the same-stage embryo, pigment cells that had already entered the aboral ectoderm were seen (Fig. 5G, J, arrowhead). At the mid-gastrula stage, pigment cells were seen in the ectoderm except the apical plate (Fig 5B, E). In the top view, it is clear that pigment cells were distributed both in the blastocoel and the aboral ectoderm (Fig. 5H, K). When the archenteron tip reached the apical plate, pigment cells had been already distributed throughout the aboral ectoderm (Fig. 5C, F and I, L). In *Ac* embryos, therefore, the position at which pigment cells enter the ectoderm is not restricted. It is important to remember that pigment cells enter only the apical plate in *Hp* embryos (Kominami *et al*., 2001). Some pigment cells were observed at the ventral side (Fig. 5L, arrowheads). This portion of the ectoderm does not differentiate into the aboral ectoderm but the anal plate ectoderm. Thus, preferential distribution of pigment cells in the aboral ectoderm was also ascertained in *Ac* embryos.

Behavior of pigment cells in *Cj* embryos was intriguing (Fig. 6); pigment cells were observed not only in the vegetal plate ectoderm but also in the blastocoel near the archenteron tip at the early gastrula stage (Fig. 6A–F). These pigment cells emanated autofluorescence with both UV- (Fig. 6B, C) and G-excitation (Fig. 6D–F). At the mid-gastrula stage, pigment cells observed on the top of the archenteron decreased in number, while pigment cells in the ectoderm increased (Fig. 6G–I). Since the total number of pigment cells did not change during these stages, some of pigment cells released from the archenteron tip might have entered the ectodermal layer nearby. Such behavior of pigment cells was observed only in *Cj*.

In the gastrulating *Tt* embryos, fluorescent pigment cells could not be detected under UV- or G-illumination. Accordingly, we could not observe the behavior of pigment

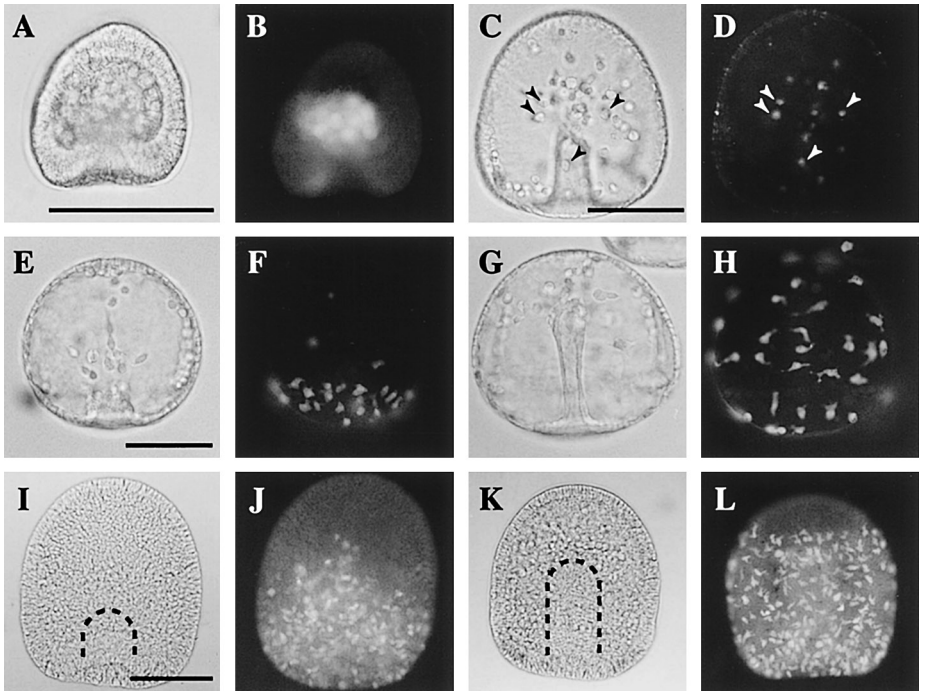

Fig. 4. *Distribution of pigment cells in gastrulating embryos.* A, B: early gastrula of *A. crassispina*. C, D: late gastrula of *M. globulus*. Arrowheads in C and D indicate some fluorescent mesenchymal cells at the same position in the blastocoel. E, F: early gastrula of *T. pileolus*. G, H: late gastrula. I, J: early gastrula of *A. manni*. K, L: late gastrula. In *A. crassispina* (A, B) and *M. globulus* (C, D), pigment cells are observed around the archenteron tip and in the blastocoel, while pigment cells are observed only in the ectodermal layer in *T. pileolus* (E–H) and *A. manni* (I–L). Dotted lines in I and K indicate the outline of the archenteron. A, C, E, G, I and K: bright field images. B. D. F. H, J and L: fluorescence images under UV-irradiation. Scale bars indicate 100 μm.

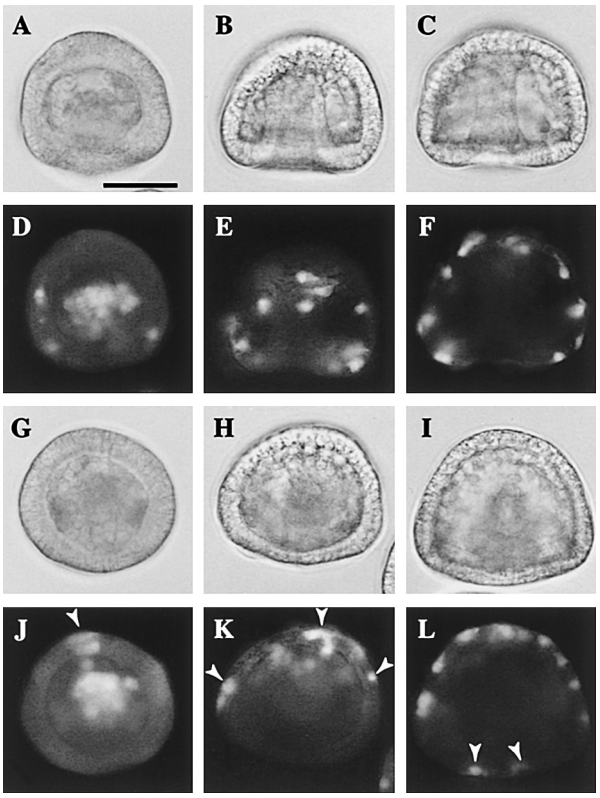

Fig. 5. *Behavior of pigment cells in A. crassispina.* A–C: bright field (frontal view). D–F: autofluorescence of pigment cells under UV-excitation (frontal view). G–I: bright field (transverse view). J–L: autofluorescence with UV-excitation (transverse view). A, D, G and J: early gastrula. Arrowheads in J and K show pigment cells that had entered the aboral ectoderm. B, E, H and K: mid-gastrula. C, F, I and L: late-gastrula. In *A. crassispina*, pigment cells are positioned at the archenteron tip at the early gastrula stage and leave the archenteron tip during gastrulation. They then migrate into the blastocoel and enter the ectodermal layer nearby. Pigment cells are preferentially distributed in the aboral ectoderm. Arrowheads in L indicate pigment cells in the border region between the oral and the anal plate ectoderm. Scale bar indicates 50 μm.

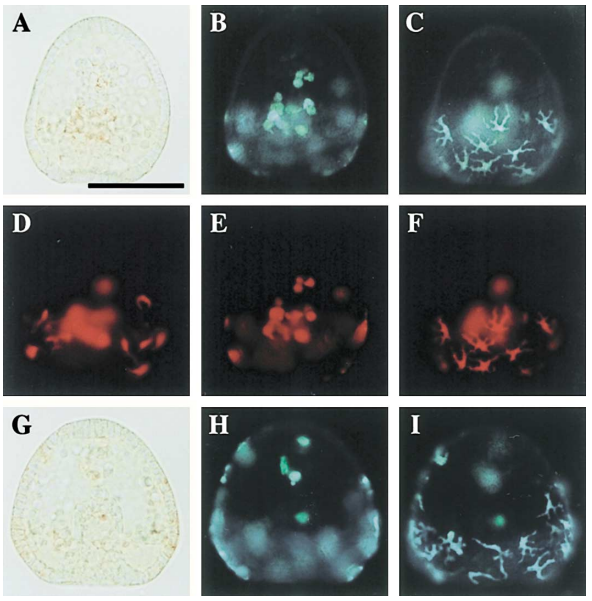

Fig. 6. *Behavior of pigment cells in C. japonicus.* A–F: an early gastrula (24 hr). G–I: a mid-to-late gastrula (28 hr). A, G: bright field. B–C and H–I: fluorescent images under the UV-irradiation. D–F: fluorescence images under the G-excitation. B, E and H: focused at the archenteron. C, F: focused at the aboral ectoderm. D, I: focused at the oral ectoderm. Few pigment cells are observed. The images of pigment cells observed under G- excitation are quite the same as those obtained with UV-excitation. In *C. japonicus* embryos, some pigment cells migrate in the dorsal ectoderm from the vegetal pole side toward the apical plate, while others are positioned on the top of the archenteron. Scale bar indicates 100 μm.

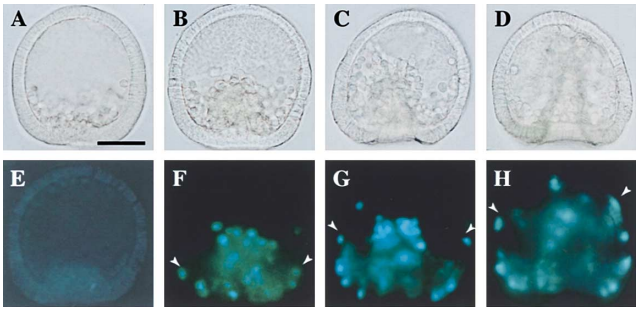

Fig. 7. *Behavior of pigment cells in hybrid embryos.* A, E: early gastrula (13 hr). B, F: mid-gastrula (15 hr). C, G: mid-to-late gastrula (17 hr). D, H: late gastrula (19 hr). A–D: bright field images. E–H: fluorescence images obtained with UV-excitation. In hybrid embryos, pigment cells are observed both on the top of the archenteron and in the ectodermal layer. Arrowheads indicate upper limit of the distribution of pigment cells, which migrate in the ectodermal layer from the vegetal pole toward the apical plate. Scale bar indicates 50 μm.

cells during gastrulation. It was the pluteus stage that pigment cells became visible upon UV- or G-irradiation. The time course of pigment synthesis in *Tt* would be much slower than in other species.

Manner of gastrulation and behavior of pigment cells in hybrid embryos

To know what degree the zygotic genome contributes the manner of gastrulation or the behavior of pigment cells, we tried to cross-fertilize the gametes obtained from *Hp* and *Sm*. As described above, the embryos of these two species show quite different manner of gastrulation and behavior of pigment cells. We succeeded in forming hybrid embryos by inseminating *Sm* eggs with *Hp* sperm, although the reverse cross-fertilization was unsuccessful.

Morphology of the hybrid embryos at the mesenchyme blastula stage resembled that of *Hp* rather than *Sm* embryos (Fig. 7A; Kominami *et al.,* 2001), with respect to the contour of embryos, thickness of the blastocoel wall and the shape of PMCs. PMCs of *Hp* embryos are globular, while those observed in *Sm* are flattened and irregular in shape. At the beginning of gastrulation, autofluorescence of pigment cells could not be detected (Fig. 7E). From the mid-gastrula stage onward (Fig. 7B, F), pigment cells became visible under UV-irradiation. It is of note that fluorescent pigment cells

became visible upon UV-irradiation in *Sm* and *Hp* embryos from the early and mid-to-late gastrula stage, respectively (Table 1; Kominami *et al*., 2001).

Fig. 7F–H illustrate the behavior of pigment cells in hybrid embryos. Interestingly, pigment cells were observed both at the tip of the invaginating archenteron and in the vegetal ectoderm. Like in *Cj* embryos, pigment cells in the ectoderm migrated from the vegetal pole side toward the apical plate. During gastrulation, pigment cells observed at the archenteron tip decreased in number, while those observed in the ectodermal layer increased.

In hybrid embryos, the archenteron elongated continuously throughout gastrulation (Fig. 8). No lag phase in the archenteron elongation could be noticed. As far as judged from the morphology of the archenteron, the archenteron cells were not rearranged during invagination processes. However, more detailed observation and experiments are needed to draw a definite conclusion.

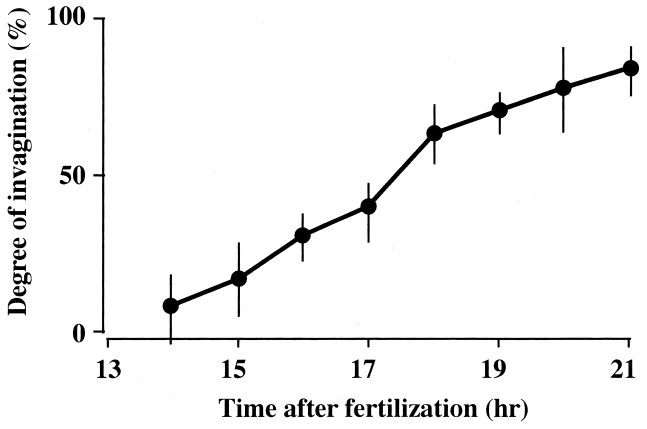

Fig. 8. *Change in the degree of invagination in hybrid embryos.* Abscissa: time after fertilization (hr). Ordinate: degree of invagination (ratio of the archenteron length to the height of the blastocoel). Each point represents the average value of 15 embryos. Degree of invagination increase gradually like in *S. mirabilis*. Vertical lines attached to the solid circles indicate S.D.

DISCUSSION

Manner of gastrulation in echinoids

Several features concerning the manner of gastrulation and behavior of pigment cells in several species of regular and irregular echinoids, including hybrid embryos, are summarized in Table 1. Some of features listed in the Table will be discussed later.

In the present study, some aspects of gastrulation processes were examined using four regular and two irregular echinoids. In two regular echinoids *Ac* and *Mg*, the archenteron elongated stepwise (Fig. 2A, B), while in two irregular echinoids *Cj* and *Am*, the archenteron elongated continuously (Fig. 2E, F). These may suggest that the manner of archenteron elongation is different between regular and irregular echinoids. In two regular echinoid *Tt* and *Tp*, however, the elongation pattern was continuous (Fig. 2C, D). Therefore, it became clear that the pattern of archenteron elongation does not reflect the current classification of echinoids.

As described, the archenteron cells are rearranged during gastrulation in popular sea urchins, such *as Strongylocentrotus purpuratus*, *Lytechinus variegatus*, or *Hp* (Ettensohn, 1985; Hardin, 1988; Kominami and Masui, 1996). As clearly shown in Figs. 3A-B, and 3E-F, archenteron cells were not rearranged during secondary invagination in *Ac* and *Mg* embryos, although their elongation pattern was stepwise (Fig. 2A, B). On the other hand, the archenteron cells were rearranged during gastrulation in *Tp* and *Cj* embryos (Fig. 3G-H and I-J), while these species show continuous elongation pattern (Fig. 2C, D). Thus, the occurrence of the rearrangement of archenteron cell again does not coincide with the pattern of archenteron elongation.

Behavior of pigment cells

It is accepted that pigment cells leave the archenteron tip at the end of gastrulation, migrate into the blastocoel and soon enter the apical plate. Some reports, however, have suggested that the timing at which pigment cells leave the archenteron is variable among sea urchins (Young, 1958; Gibson and Burke, 1985). Further, from the observation on PMC-deficient embryos, Ettensohn and McClay (1988) found that in *L. variegatus* pigment precursor cells left the archenteron during primary invagination and entered the ectoderm nearby, i.e., entry site of pigment cells was not limited to the apical plate.

As revealed in the present study, pigment cells in *Ac* embryos began to leave the archenteron from the early phase of invagination, and soon entered the aboral ectoderm nearby (Fig. 5), like in *L. variegatus*. On the other hand, quite different behavior was seen in *Tp* and *Am* embryos (Fig. 4). Pigment cells migrated from the vegetal plate toward the apical plate through the aboral ectoderm, without entering the blastocoel. This behavior of pigment cells is quite the same as that observed in *Sm* (Kominami *et al*., 2001).

In *Am* embryos, a huge number of pigment cells were observed (Table 1). Previously, we show that the number of pigment cells correlates the number of cell division cycles of pigment founder cells, while the number of pigment founder cells is not so different among species (Kominami, 2000; Kominami and Takata, 2003, Takata and Kominami, 2004). It is probable that the number of division cycles of pigment founder cells is larger in *Am* than that in other species, because the eggs of *Am* are quite large (nearly 250 μm in diameter).

Now, it is clear that the behavior of pigment cells closely correlate the manner of gastrulation. In the embryos that show the stepwise invagination, pigment cells are positioned at the archenteron tip and leave the archenteron during gastrulation (Fig. 2A, B, Fig. 4A–D, Fig. 5). In contrast, in the embryos that show continuous invagination, pigment cells

do not enter the blastocoel, and begin to migrate from the vegetal pole side toward the animal pole through the aboral ectoderm. Only one exception was seen in *Cj* embryos; some of pigment cells migrated directly from the vegetal plate toward the animal pole, while some were positioned at the archenteron tip (Fig. 6).

The localization of pigment cells in the aboral ectoderm was ascertained in all species examined. As is well known, the composition of extracellular matrix (ECM) is different between the oral and aboral ectoderm (Coffman and McClay, 1990; Hardin *et al.,* 1992; Davidson *et al.,* 1998). Such difference in ECM components between oral and aboral ectoderm may be prevailing in a variety of echinoids, and bring about the localization of pigment cells.

Manner of gastrulation and behavior of pigment cells in hybrid embryos

It has been known that in hybrid embryos, the developmental characteristics observed during early phase of development, such as the cleavage intervals and the timing of the onset of gastrulation, reflect the maternal properties, and that paternal characteristics become to be noticed from the gastrula stage onward (Giudicce, 1986). In fact, time schedule of cleavages in hybrid embryos resembled that observed in *Sm*, and gastrulation started almost simultaneously with *Sm* embryos (data not shown). Interestingly, some paternal characteristics were detected even before the onset of gastrulation. As stated in the Results, the external morphology of the hybrid mesenchyme blastulae was very similar to that of *Hp* embryo (Fig. 7A).

The most interesting feature in hybrid embryos is the appearance of pigment cells at the invaginating archenteron tip (Fig. 7). The behavior of pigment cells in hybrid embryos was quite the same as that observed in *Cj* embryos. The archenteron elongated continuously as well (Fig. 8). The rearrangement of archenteron cells, however, did not seem to occur in hybrid embryos. This suggests that the behavior of pigment cells is irrelevant to the occurrence of the rearrangement of archenteron cells. During the process of evolution, some species in both regularia and irregularia must have acquired independently the cellular basis that caused the rearrangement of archenteron cells. It is intriguing to learn how *Brachyury* relates the rearrangement of archenteron cells, since *Brachyury* looks to be deeply involved in the invagination processes (Gross and McClay, 2001; Rast *et al.,* 2002).

Divergence in the manner of gastrulation and behavior of pigment cells

Three features of archenteron elongation would define the manner of gastrulation; whether the archenteron elongates stepwise or continuously, whether the archenteron reaches the apical plate or not, and whether the archenteron cells are rearranged or not during the latter phase of gastrulation (Fig. 9A). It is generally thought that echinoids had been derived from starfishes during evolution. In starfishes, such as *Asterina pectinifera*, the archenteron elongates continuously, does not reach the animal pole, and the archenteron cells are not rearranged during gastrulation (Kuraishi and Osanai, 1992). Interestingly, quite the same characteristics of archenteron elongation are seen in *Am*. It is naturally supposed that the manner of gastrulation in the ancestral echinoids would be similar to that observed in *Am* embryos.

As well as the manner of gastrulation, the behavior of pigment cells is also variable among echinoids (Fig. 9B). It is reasonable to suppose that the behavior of pigment cells seen in *Am* is also the prototype. Then, how does the variety of the behavior of pigment cells bring about? At the mesenchyme blastula stage, precursors of pigment cells are organized into the vegetal plate as epithelial cells (Ruffins and Ettensohn, 1993, 1996). As well as PMCs, pigment precursor cells should undertake the epithelial-to-mesenchyme transition (EMT, Peterson and McClay, 2003), whereas destination of pigment cells is not the blastocoel but the ectoderm layer. If pigment precursor cells undertake EMT before the onset of gastrulation, they would begin to migrate from the vegetal pole side toward the animal pole through the ectoderm. On the contrary, pigment cells would stay at the archenteron tip, if EMT does not occur until the completion of primary invagination. After EMT, they would migrate into the blastocoel and then enter the ectoderm. The occurrence of EMT might have been delayed during the process of evolution, resulting in the appearance of pigment cells at the archenteron tip. Notably, the timing at which pigment cells become visible is later in regular echinoids than irregular echinoids (Table 1). The behavior of pigment cells observed in *Cj* embryos might reflect a transitional phase.

Fig. 9C shows the current classification of echinoids examined in this study. In the figure, symbols of the events that modified the manner of gastrulation (Fig. 9A) and behavior of pigment cells (Fig. 9B) are superimposed. As seen in the figure, it is necessary to assume that the same event occurred independently in several branches. On the other hand, Fig. 9D shows a putative classification, in which attention is paid so that the number of the events would be less as far as possible. Although 14 events are superimposed in the former (Fig. 9C), 9 events are enough to explain the divergence in the manner of gastrulation and behavior of pigment cells observed in 9 species listed (Fig. 9D). The most conspicuous difference between the two schemes is the position of *Tp*. In Fig. 9D, *Tp* (regularia) belongs to the group in which most of the irregularians are classified. As has been sometimes pointed, evolution of developmental pathways does not necessarily reflect the current classification, which is primarily based on the morphological characteristics of adult animals (Wray and Bely, 1994).

It should be reminded that pigment cells are bottle cells at least in *Em* (Takata and Kominami, 2004). During evolution, pigment cells are thought to have acquired the ability to act as bottle cells in some species of regular echinoids. It is highly probable that appearance of bottle cells would have

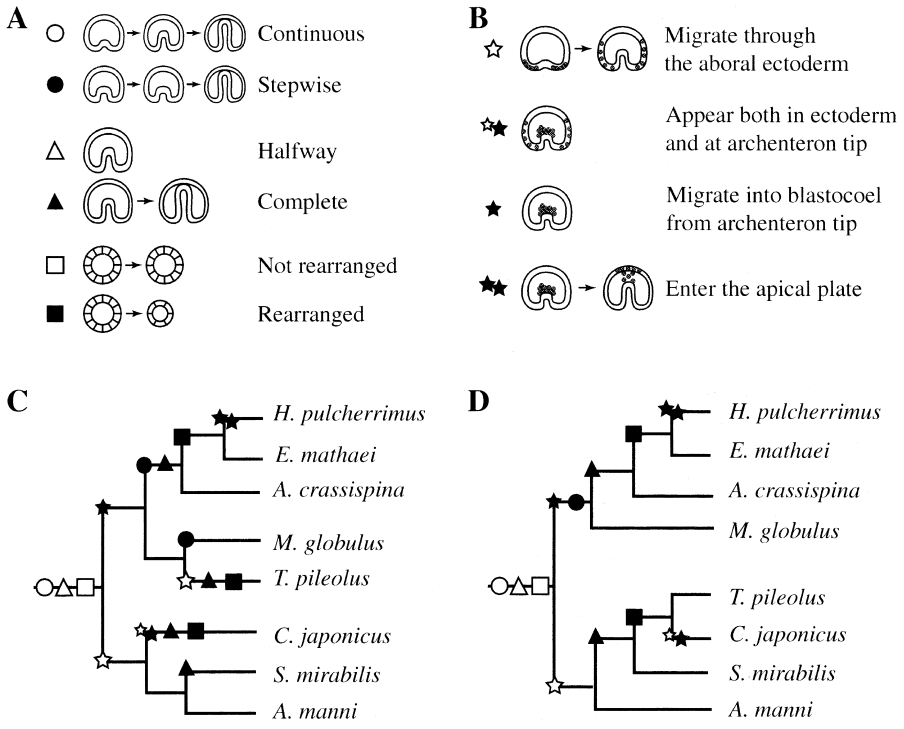

Fig. 9. *Classification of echinoids based on the events that had modified the manner of gastrulation and the behavior of pigment cells.* A: events that caused the variance in the manner of gastrulation. B: events that modified the behavior of pigment cells. C: a current taxonomic branch of echinoids examined in this study. Symbols of events are superimposed. D: a putative classification of echinoids based on the results obtained in the present study. Number of events necessary for explaining the divergence in echinoids is less in this illustration than that in C. *T. toreumaticus* is excluded from the figures, since the behavior of pigment cells could not be observed during gastrulation, due to the lack of autofluorescence. In C and D, hybrid embryos are not included, since it could not be determined precisely whether the rearrangement of archenteron cells occurred or not.

changed the pattern of archenteron elongation from continuous to stepwise. This possibility should be tested in the near future by examining whether bottle cells are observed in the vegetal plate before the onset of gastrulation or not. As well as the molecular approaches, detailed observation of the developmental pathways would be helpful for the further understanding the evolutionary divergence seen in echinoids.

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