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ハスノハカシパン胚における細胞質の局在と頂板形成

Localized cytoplasm and apical organ formation in embryos of the sand dollar *Scaphechinus mirabilis*

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ABSTRACT

The animal pole region of the sea urchin larva is composed of the thickened animal plate, and the apical ganglion formed by several serotonergic cells. Recent studies have revealed many genes involved in the differentiation and construction of these structures. However, the cellular basis of specification process is largely unknown. To understand how these structures are specified, we tried to clarify the properties of animal hemisphere by manipulative methods. The isolation experiments revealed that some cytoplasmic factors responsible for the thickening of the ectodermal epithelium form a gradient from the animal pole toward the equator. The deletion experiments showed that an_1 blastomeres are required for the formation of animal plate and apical ganglion. Recombination and transplantation experiments suggested that the presumed thickening factors are easily inactivated by the vegetal signal, and that a low concentration of thickening factors promotes the differentiation of serotonergic cells, while the factors inhibit serotonergic cell differentiation at higher concentrations. Further, pulse treatment with animalizing or vegetalizing reagent showed that the specification process terminates during the mesenchyme blastula stage. Based on these results, we propose a model that explains the experimentally caused changes in the size of animal plate and in the width of apical ganglion.

Key words: *animal plate, apical ganglion, cell signaling, cytoplasmic factor, sea urchin*

Introduction

In the sea urchin gastrula, cells near the animal pole become thickened and form a placode called animal plate. Animal plate cells have long apical tuft cilia, which are thought to serve as a sensory organ. As is well known, the animal half isolated from the 8-cell stage embryo develops into a dauerblastula, a spherical hollow blastula with an expanded apical-tuft-forming area (Hörstadius, 1973: Maruyama *et al*., 1985). This indicates that the ectoderm patterning requires some signals from the vegetal pole side (Hardin and Armstrong, 1997: Wikramanayake *et al*., 1997), and that the signal restricts the apical-tuft forming ability in the animal hemisphere except the animal pole region.

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Such a restriction signal, designated “vegetal signal” in Yaguchi *et al*. (2006), is mediated through canonical Wnt signaling pathway, and is beta-catenin-dependent; fertilized eggs injected with delta-cadherin mRNA give rise to dauerblastulae with an expanded animal plate. A dominant negative type of glycogen synthase kinase (GSK) or dishevelled mRNA also induces a similar phenotype (Yaguchi *et al*., 2006: Leonard and Ettensohn, 2007). Wnt8, which is initially expressed in micromeres, is a well-known member of Wnts in the sea urchin embryo (Wikramanayake *et al*., 2004). However, this paracrine factor does not seem to be involved in this restriction, since its expression is limited to the vegetal hemisphere. In fact, the animal plate forms normally even if micromeres are removed (Yaguchi *et al*., 2008). Some kinds of Wnts other than Wnt8 (Croce *et al*.,

2006) would be involved in the vegetal signal. The vegetal signal is suggested to originate from the subequatorial region (Sasaki and Kominami, 2008). Notably, numerous serotonergic cells appear in the embryo injected with delta-cadherin mRNA (Yaguchi *et al*., 2006). Thus, the vegetal signal also restricts the differentiation of serotonergic cells.

By the early pluteus stage, several serotonergic cells form a neuroplexus (apical ganglion) at the aboral side near the animal plate (Burke, 1978: Nakajima *et al*., 1993: Yaguchi *et al*., 2000: Takacs *et al*., 2004). This apical ganglion is suggested to be a prototype of central nervous system developed in vertebrates (Poustka *et al*., 2004). Yaguchi *et al*. (2006) suggested that the Nodal signaling, which plays an important role in establishing the oral/aboral axis (Duboc *et al*., 2004), is also involved in the differentiation of serotonergic cells. Over-expression of Nodal, whose signal is mediated by Smad (Yaguchi *et al*., 2007), suppresses serotonergic cell differentiation. Conversely, the molecules that expand the aboral ectoderm, such as BMP (Angerer *et al*., 2000), promote the differentiation of neural cells (Yaguchi *et al*., 2006). Interestingly, the presumptive animal plate cells are incompetent to the molecules involved in the oral/aboral specification; over-expression of those molecules does not affect the size of animal plate (Yaguchi *et al*., 2006).

This stability of the animal plate suggests that blastomeres in the animal hemisphere are not homogeneous. Sasaki and Kominami (2008) showed that the an_1 tier of blastomeres contains the cytoplasmic factors necessary for thickening of the animal plate; the animal plate does not thicken if all the an_1 blastomeres are removed. Further, the ectoderm patterning along the oral/aboral axis requires the subequatorial cytoplasm, as revealed by micromanipulations (Kominami *et al*., 2006). The cytoplasmic composition of sea urchin eggs would be more complicated than generally thought. To understand the cellular basis of of animal plate and apical ganglion formation, it is necessary to reveal further the properties of blastomeres in the animal hemisphere.

In this study, an_1 and an_2 tiers of blastomeres were isolated from the 32-cell stage embryos to know whether developmental potencies are the same between them. Second, various numbers of an_1 blastomeres were removed from 32-cell stage embryos, to examine how many an_1 blastomeres are necessary for the animal plate formation. Third, the an_1 tier of blastomeres was combined directly with the vegetal hemisphere to know whether the animal plate forms when the an_1 tier is exposed to more intensive restriction signal. Fourth, the an_1 blastomeres were transplanted ectopically to know whether their fate is altered or not. Finally, embryos were pulse-treated with LiCl or $ZnSO_4$, to know whether the size of animal plate affects the apical ganglion formation. In this report, the animal plate and the apical ganglion will be collectively called an “apical organ”.

Materials and methods

Animals and embryos

Adults of the sand dollar *Scaphechinus mirabilis* were collected during their breeding seasons, and kept in aquaria supplied with circulating seawater. Gametes and fertilized eggs were obtained with the standard method. Millipore-filtered seawater (MFSW) supplemented with antibiotics (Penicillin G potassium, 100 units/ml: Streptomycin Sulfate, 50 μg/ml) was used throughout experiments. Embryos were reared at 18 ˚C.

Micromanipulation

Unfertilized eggs were inseminated in MFSW containing 1 mM amino-triazole (Showman and Foerder, 1979). About 10 minutes after insemination, the softened fertilization envelopes were removed using a nylon mesh. About 10 minutes prior to manipulation, early cleavage stage embryos were transferred into a culture dish containing calcium-free artificial seawater (CaFSW). After cell contacts were loosened, we performed micromanipulation (separation, deletion, recombination, and transplantation) under an inverted microscope, using a hand-pulled glass needle. To avoid the damage caused by manipulation, culture dishes were coated with 1.5 % agar dissolved in CaFSW. The manipulated embryos were transferred into wells of a cell culture plate (96 wells) filled with MFSW. Control embryos and manipulated embryos were observed at 24 hours post

fertilization (hpf), unless otherwise noted.

Construction of an_2*-depleted embryos*

An aliquot of fertilized eggs was stained with 50 μg/ml tetramethyl-rhodamine isothiocyanate (RITC, Sigma-Aldrich, St Louis, MO, USA) for 20 minutes, and then rinsed four times with MFSW. Immediately after embryos reached the 32-cell stage, they were transferred into a culture dish containing CaFSW. The an_1 tier and the vegetal hemisphere were isolated from the stained embryos and non-stained embryos, respectively, and were combined.

Observation of apical tuft

To observe apical tuft, intact or manipulated embryos were fixed with 1 % glutaraldehyde, which did not cause deciliation unlike formaldehyde. Fixed embryos were mounted on a glass slide, and covered with a cover slip. The specimens were oriented by sliding the cover slip so that they would be observed along the animal-vegetal axis, and examined under a microscope equipped with differential interference contrast (DIC) optics. The cells having long apical tuft cilia, whose length is more than double the length of normal cilia, are defined as animal plate cells. Like in a previous report (Sasaki and Kominami, 2008), the number of animal plate cells was counted on a focal plane, and was employed to represent the size of animal plate.

Detection of serotonergic cells

Embryos were fixed with 4 % paraformaldehyde (dissolved in MFSW) for 10 minutes, and rinsed three times (10 minutes each) with phosphate-buffered saline supplemented with 1 % Tween-20 (PBST). The fixed specimens were incubated with rabbit anti-serotonin polyclonal antibody (Sigma-Aldrich, 1:1,000 dilution) overnight. After rinsing three times with PBST, the specimen was reacted with fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G polyclonal antibody (Sigma-Aldrich, 1:50 dilution) for two hours with occasional agitation, and rinsed three times with PBST.

Treatments with chemicals

Embryos were treated with 30 mM LiCl or 1 mM $ZnSO_4$ every 4 hours after fertilization. At the end of treatment, embryos were rinsed three times with MFSW. The $ZnSO_4$-treated embryos were further incubated in MFSW containing 0.1 mM EDTA-OH to chelate the remnant Zn^{2+} for an hour (Tokuoka *et al*., 2002).

Cell tracing

Lucifer Yellow CH (Sigma-Aldrich) was iontophoretically injected into one of blastomeres at the 8-cell stage after the method described before (Kominami, 1988).

Results

Morphological difference between the blastulae derived from the an_1*- and* an_2*-tiers of blastomeres*

By 24 hpf, *S. mirabilis* embryos reached the late gastrula stage (Fig. 1A). The ectoderm near the animal pole (between arrowheads) became thicker, compared with the lateral blastocoel wall. In one half of the thickened region, cells near the animal pole formed long apical tuft cilia (indicated by a bracket).

We first isolated the animal halves at the 8-cell and 16-cell stages, to know its ability to form the animal plate in the absence of the vegetal hemisphere (Fig. 1B). The isolated animal halves developed into two types of spherical hollow blastulae; one was the dauerblastula composed of almost homogeneously thickened blastocoel wall (Figs. 1C, E), and the other was the blastula that showed gradual decrease in the thickness of the blastocoel wall toward the vegetal pole side (Figs. 1D, F). Appearance frequencies of the former type blastulae were 44 % (n = 27) and 37 % (n = 27), when separated at the 8-cell and 16-cell stages, respectively.

Figure 2 shows the thickness of the thickest region (t_1) and the thickness of the region opposite to it (t_2) in the obtained spherical hollow blastulae. The t_1 valuses in the animal half embryos derived from the animal hemisphere isolated at the 8-cell stage and the 16-cell stages were smaller than the thickness of the animal plate (ap). On the other hand, the t_2 valuses in the animal half embryos were larger than the

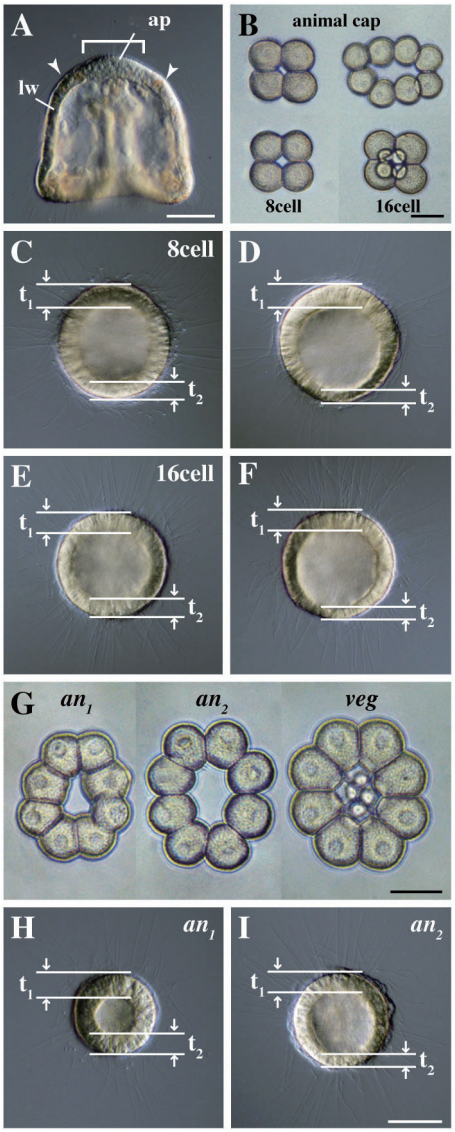

Figure 1. Development of animal half, an_1-tier, and an_2-tier of blastomeres

A; an intact late gastrula observed at 24 hours postfertilization (hpf). Animal pole region (between arrowheads) becomes thickened. A bracket indicates the region where long apical tuft cilia are formed. **B**; Two embryo fragments at the left side were isolated from animal (top) and vegetal (bottom) hemispheres at the 8-cell stage. Two embryo fragments at the right side were obtained from the 16-cell stage embryo. **C**, **D**; animal half embryos derived from the animal hemisphere separated at the 8-cell stage. t_1, t_2; thickness of the thickest and thinnest regions, respectively. **E**, **F**; half embryos derived from the animal hemisphere separated at the 16-cell stage. **C**, **E**; animal half embryos, in which the difference between t_1 and t_2 is small. **D**, **F**; the difference between t_1 and t_2 is conspicuous. **G**; left, middle and right are an_1 tier, an_2 tier and vegetal hemisphere (veg) isolated at the 32-cell stage, respectively. **H**; an an_1-blastula. **I**; an an_2-blastula. All scale bars indicate 50 μm. The scale bar in **I** is common to **C**-**F** and **H**.

thickness of the lateral blastocoel wall (lw). The difference between "ap" and t_1, or between "lw" and t_2 might be ascribed to the formation of a blastula composed of a smaller number of cells. However, direct comparison is possible between the embryos derived from animal hemispheres separated at the 8-cell stage and 16-cell stage. The thickest region of the blastula derived from the animal halves (t_1 indicated in Figs. 1C-F) became thinner when isolated at the 16-cell stage than the 8-cell stage ($P < 0.05$). In contrast to this, the thickness of the thinnest region (t_2 indicated in Figs. 1C-F) did not change significantly. These suggest that restriction process of the thickening ability of animal blastomeres starts as early as the 16-cell stage.

Next, we tried to isolate an_1 and an_2 tiers of blastomeres at 32-cell stage (Fig. 1G). The isolated tiers of blastomeres underwent cleavages with a normal time schedule, and gave rise to blastula-like structures (Figs. 1H, I). In a small dauerblastula derived from the an_1-tier of blastomeres (an_1-blastula), whole blastocoel wall thickened, and the difference between t_1 and t_2 was not so conspicuous. In contrast, the t_1 was considerably larger than t_2 in the blastula derived from the an_2-tier of blastomeres (an_2-blastula). Consequently, the an_2-blastula was larger than the an_1-blastula (compare Fig.

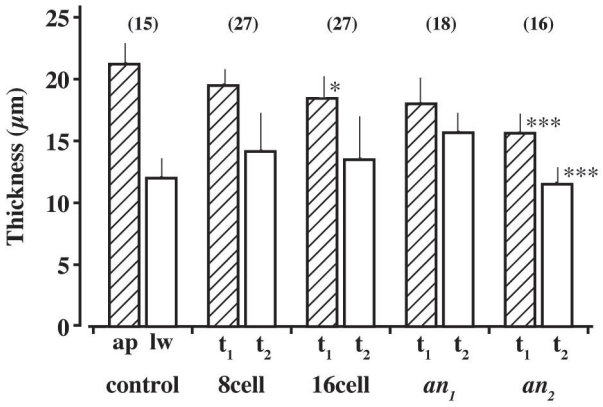

Figure 2. Thickness of blastocoel wall in control embryos and manipulated embryos

Abscissa; type of embryos. Ordinate; thickness of the blastocoel wall (μm). ap; animal plate (hatched). lw; lateral blastocoel wall (open). t_1, t_2; thickness of the thickest (hatched) and the thinnest region (open), respectively. Attached vertical thin lines indicate SD. The t_1 and t_2 values were compared (Sudent's t-test) between "8-cell" and "16-cell", and between "an_1" and "an_2". *: $P < 0.05$. **: $P < 0.01$. ***: $P < 0.001$. The numbers of examined embryos are given in parentheses.

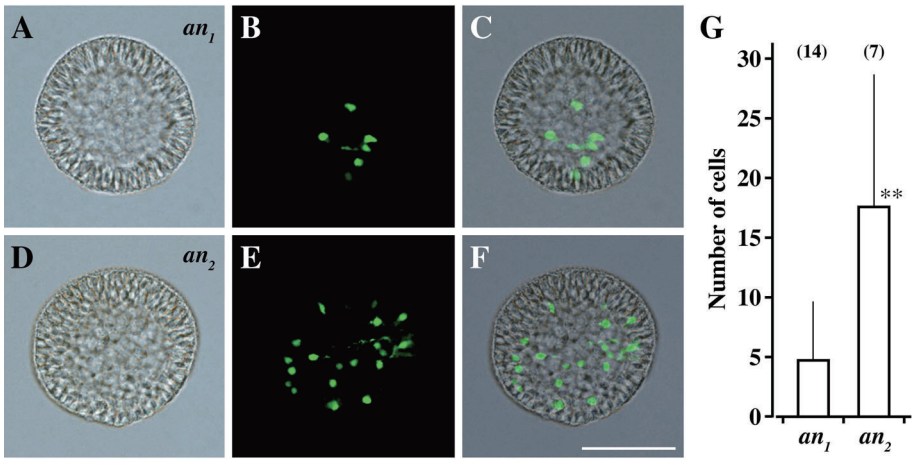

Figure 3. Serotonergic cells formation in the an_1- and an_2-blastulae

A-**C**; an an_1-blastula. **D**-**F**; an an_2-blastula. **A**, **D**; bright field images. **B**, **E**; fluorescence images. **C**, **F**; merged images of **A**, **B** and **D**, **E**, respectively. Animal pole side is at the top. The scale bar in **F** is common to **A**-**E** and indicates 50 μm. **G**; the numbers of serotonergic cells in the an_1 and an_2-blastulae. Some an_1-blasulae differentiated no serotonergic cell. Attached vertical thin lines indicate S.D. ** ; the difference between "an_1" and "an_2" is statistically significant ($P < 0.01$). The numbers of examined embryos are given in parentheses.

1I with Fig. 1H). Interestingly, the t_2 of an_1-blastulae was almost the same as the t_1 in an_2-blastulae (Fig. 2). Further, the t_2 of the an_2-blastula was comparable to the thickness of the lateral blastocoel wall in control embryos. Thus, the thickening ability of cells in the animal hemisphere formed a gradient from the animal pole toward the equatorial region.

Differentiation of serotonergic cells in an_1- *and* an_2-*blastulae*

To examine whether the potential to form serotonergic cells was different between an_1 and an_2 tiers of blastomeres, we isolated them from the 32-cell stage embryos, and detected serotonergic cells at 36 hpf (Fig. 3). In an an_1-blastula, several serotonergic cells were observed in a cluster (Figs. 3A-C). Notably, a larger number of serotonergic cells were scattered in the an_2-blastula (Figs. 3D-F). In both blastulae, serotonergic cells were preferentially distributed at the vegetal pole side, where the blastocoel wall was thinner.

The averaged numbers of serotonergic cells in an_1- and an_2-blastulae were 4.7 and 17.6, respectively (Fig. 3G). Of 14 an_1-blastulae, six did not differentiate serotonergic cell at all. On the other hand, all the an_2-blastula differentiated serotonergic cells more or less. As the large standard deviation indicates, the number was fairly variable. In an extreme case, 31 serotonergic cells were observed. If the animal hemisohere had not been separated into an_1 and an_2 tiers of blastomeres, it differentiates about 20 serotonergic cells in most cases (data not shown).

Effects of an_1 *blastomere depletion on the apical organ formation*

As described above, the animal plate does not thicken if the an_1 blastomeres are removed (Sasaki and Kominami, 2008). To study more precisely the role of an_1 blastomeres in the animal plate formation, different numbers of an_1 blastomeres were removed from the 32-cell stage embryo. Figure 4A shows an embryo from which two an_1 blastomeres were removed. The manipulated embryos (Fig. 4C) developed into late gastrulae morphologically similar to the intact embryo (Fig. 4B). If more than six an_1 blastomeres were removed, the manipulated embryos showed a delay in the outgrowth of post-oral arms (Fig. 4D). The width of the animal plate (Figs. 4B-D, indicated by brackets) did not differ between control embryos and manipulated embryos. However, the thickness of the animal plate decreased, especially when six or eight (all) an_1 blastomeres had been removed. Hereafter, the embryo from which all an_1 blastomeres were removed would be called an "an_1-depleted embryo".

To quantify the morphological changes caused by the removal of an_1 blastomeres, we measured the width of animal plate, thickness of the animal plate, thickness of

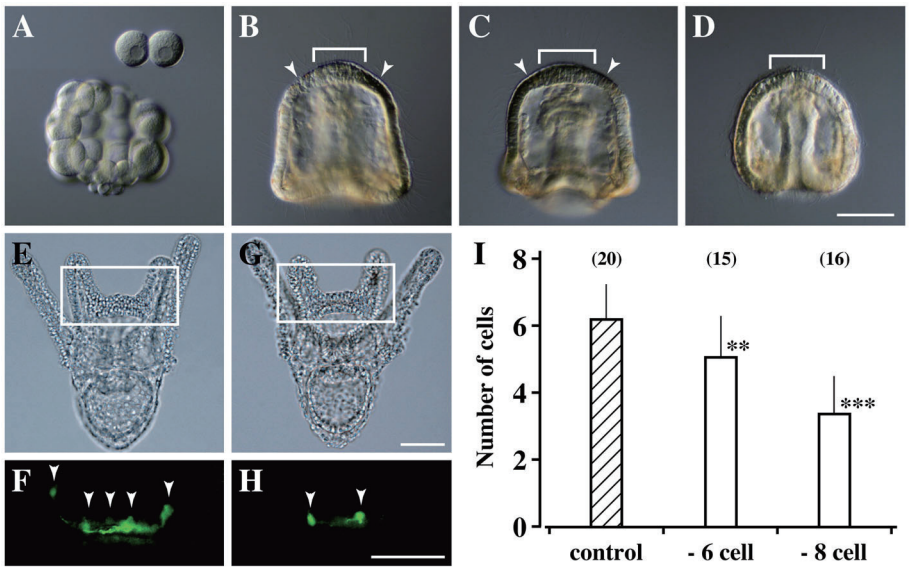

Figure 4. Apical organ formation in the embryo from which a pair number of *an1* blastomeres had been removed

A; an embryo from which two *an1* blastomeres had been removed at the 32-cell stage. **B**-**D**; control embryos and manipulated embryos at 24 hpf. **B**; a control embryo. **C**; an embryo from which two *an1* blastomeres had been removed. The animal plate became thinner slightly. **D**; six *an1* blastomeres had been removed. Thickening of the animal plate is scarcely noticed. Brackets indicate the animal plate with long apical tuft cilia. Arrowheads indicate the boundary of thickening region. **E**, **G**; manipulated embryos observed at 48 hpf. **E**; six *an1* blastomeres had been removed. **F**; fluorescence image of the region indicated with a white rectangle in **E**. **G**; all *an1* blastomeres had been removed. **H**; fluorescence image of the region indicated with a white rectangle in **G**. Arrowheads in **F** and **H** indicate the position of cell bodies of serotonergic cells. The scale bar in **D** is common to **A**-**C**. The scale bar in **G** is common to **E**. The scale bar in **H** is common to **F**. All bars indicate 50 μm. **I**; the numbers of serotonergic cells in control embryos, and in manipulated embryos. Attached vertical thin lines indicate S.D. **; the difference between control embryos and manipulated embryos is statistically significant ($P < 0.01$). ***; $P < 0.001$. The numbers of examined embryos are given in parentheses.

lateral blastocoel wall, length of apical tuft, and length of normal beating cilia (Table 1). The width of animal plate, the number of animal plate cells observed in a focal plane, was not drastically altered by the removal of *an1* blastomeres, although the decrease is statistically significant when six or eight *an1* blastomeres were removed. The animal plate became thinner as a larger number of *an1* blastomeres had been removed. It is interesting to note that the thickness of animal plate in *an1*-depleted embryos is similar to the thickness of lateral blastocoel wall of control embryos. The thickness of lateral blastocoel wall was almost the same between control embryos and manipulated embryos. The length of apical tuft became shorter as the number of removed *an1* blastomeres was increased, while the length

Table 1. Morphological characteristics of the late-gastrula stage embryo from which *an1* blastomeres were removed

	Number of animal plate cells	Thickness of animal plate (μm)	Thickness of lateral wall (μm)	Length of apical tuft (μm)	Length of cilia (μm)
Control	13.7 ± 1.6§	21.3 ± 1.4	12.0 ± 1.3	58.5 ± 4.2	24.5 ± 2.4
-2 *an1*	12.7 ± 1.2	21.4 ± 2.7	12.7 ± 1.9	58.7 ± 6.3	23.4 ± 2.5
-4 *an1*	12.9 ± 2.0	19.9 ± 1.8*	11.6 ± 1.8	57.4 ± 8.7	21.1 ± 3.3**
-6 *an1*	11.9 ± 1.3**	17.5 ± 1.9***	12.0 ± 1.4	52.3 ± 7.7*	22.4 ± 3.1
-8 *an1*	12.1 ± 1.1**	13.4 ± 2.0***	10.6 ± 1.0**	51.1 ± 5.5***	22.9 ± 3.7

§: average and standard deviation were obtained by examining 15 embryos. *; the difference in the averages between control embryos and manipulated embryos is statistically significant ($P < 0.05$). **; $P < 0.01$. ***; $P < 0.001$.

of beating cilia was similar between intact embryos and manipulated embryos.

The number of serotonergic cells decreased if six (Figs. 4E, F) or all (Figs. 4G, H) an_1 blastomeres had been removed. Figure 4I shows the numbers of serotonergic cells differentiated in control embryos and in manipulated embryos. In control embryos, about six cells were observed. The number decreased to about one half in an_1-depleted embryos, although almost the same number of serotonergic cells was observed in embryos from which two or four an_1 blastomeres had been removed (data not shown).

Apical organ formation in an_2*-depleted embryos*

If the an_2 tier of blastomeres were absent, the vegetal signal might reach the presumptive animal plate cells more quickly. To test this, we combined an_1 tier of blastomeres directly with vegetal hemisphere (Figs. 5A, B). By 24 hpf, such an_2-depleted embryos developed into late gastrulae with a slight delay. In the embryo shown in Figures 5C-E (frontal view), the descendants of an_1 blastomeres were distributed from the animal pole to the equatorial region, compensating the lack of an_2 tier of blastomeres.

Morphological characteristics of the an_2-depleted embryo are listed in Table 2. The number of animal plate cells decreased by about three. This decrease was statistically significant. The thickness of animal plate also significantly decreased. Difference in the thickness of lateral blastocoel wall between control embryos and an_2-depleted embryos was small. The length of apical tuft was significantly shorter in combined embryos.

At 48 hpf, an_2-depleted embryos reached the pluteus stage (Fig. 5F). In such larvae, only a small number of serotonergic cells were observed (Fig. 5G). The averaged number of serotonergic cells was about two (Fig. 5H). Of 15 an_2-depleted embryos examined, 4 embryos did not differentiate serotonergic cells at all.

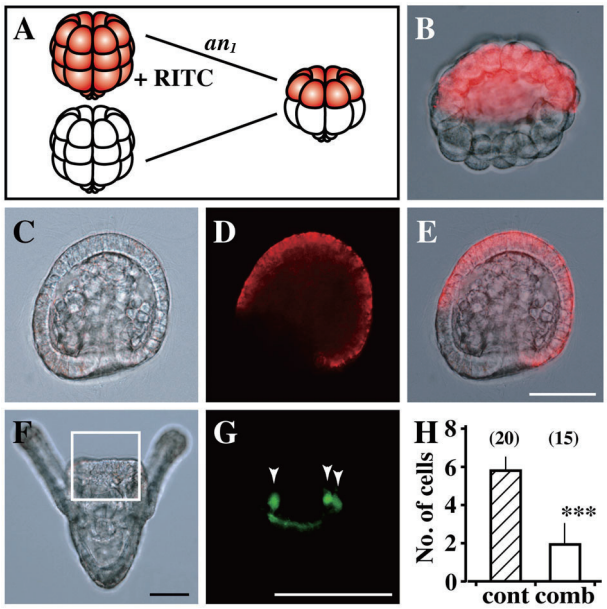

Figure 5. Apical organ formation in an_2-depleted embryos

A; a diagrammatic representation of chimera formation. The an_1 tier stained with RITC was combined with a non-labeled vegetal hemisphere at the 32-cell stage. **B**; an an_2-depleted embryo about one hour after combination. **C**-**E**; observed at 24 hpf. **C**; bright field image. **D**; fluorescence image. **E**; a merged image of **C** and **D**. Descendants of the an_1 tier extend to the most part of ectoderm. **F**; an an_2-depleted embryo at 48 hpf. **G**; fluorescence image of a region indicated with a white square in **F**. Arrowheads indicate the cell bodies of serotonergic neurons. The scale bar in **E** is common to **B**-**D**. All scale bars indicate 50 μm. **H**; the numbers of serotonergic cells observed in control embryos (cont) and an_2-depleted embryos (comb). Attached vertical thin bars indicate S.D. The numbers of examined embryos are given in parentheses. ***: the difference between control embryos and manipulated embryos is statistically significant ($P < 0.001$).

Table 2. Morphological characteristics of an_2-depleted embryos

	Number of animal plate cells	Thickness of animal plate (μm)	Thickness of lateral wall (μm)	Length of apical tuft (μm)	Length of cilia (μm)
Control	12.9 ± 1.4§	21.9 ± 1.4	10.8 ± 1.0	59.2 ± 3.5	22.3 ± 2.0
Combined	10.0 ± 1.5***	18.1 ± 1.5***	11.6 ± 0.9*	40.0 ± 12.7***	22.4 ± 2.2

§: average and standard deviation were obtained by examining 15 embryos. *; the difference in the averages between control embryos and manipulated embryos is statistically significant ($P < 0.05$). ***; $P < 0.001$.

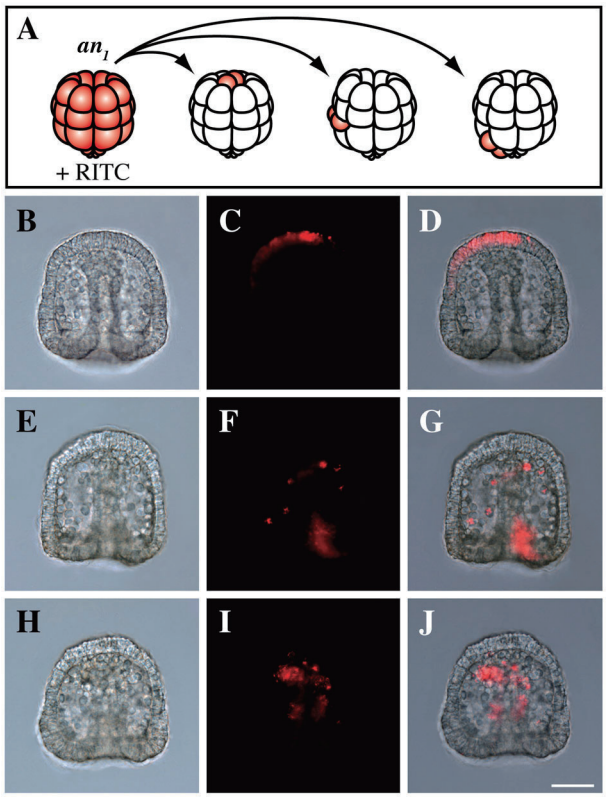

Figure 6. Transplantation of two *an1* blastomeres to whole embryo at the 32-cell stage

A; a diagrammatic representation of transplantation experiments. The donor embryos were stained with RITC, and two *an1* blastomeres isolated. Then, they were transplanted to various positions of whole embryo. **B**-**D**; transplanted to the animal pole. **E**-**G**; transplanted to the equator. **H**-**J**; transplanted to vegetal pole region. **B**, **E**, **H**; bright field images. **C**, **F**, **I**; fluorescence images. **D**, **G**, **J**; merged images. The scale bar in **J** is common to **B**-**I** and indicates 50 μm.

Transplantation of an$_1$ *blastomeres to ectopic positions*

To know whether *an1* blastomeres exert their thickening and apical tuft forming capacities even at ectopic positions, two *an1* blastomeres were transplanted to a 32-cell stage host embryo (Fig. 6A). The reasons why two blastomeres were transplanted are as follows. First, a larger number of *an1* blastomeres were not successfully built in the blastocoel wall of host embryos. Second, it was rather easy to isolate a pair of *an1* blastomeres due to the existence of cytoplasmic bridge between sister blastomeres. Third, even two *an1* blastomeres cause a considerable thickening of animal plate at the original position (Table 1, compare the thicknesses of animal paltes of -6 *an1* and -8 *an1*). By 24 hpf, the manipulated embryos developed into late gastrulae without delay (Figs. 6B, E, H).

A normal-sized animal plate was formed when two *an1* blastomeres had been transplanted near the animal pole (Figs. 6B-D); expansion of the animal plate was not noticed. When *an1* blastomeres were transplanted to the equator (Figs. 6E-G), they would receive Wnt8 that induces the endodermal tissue (Wikramanayake *et al*., 2004). The descendants were detected at the bottom of archenteron and some secondary mesenchyme cells (Figs. 6E-G). If the *an1* blastomeres had been transplanted near the vegetal pole, it would be exposed to both Wnt8 and delta, a signal molecule that specifies the secondary mesenchyme lineage (Sherwood and McClay, 1999: Sweet *et al*., 2002). Expectedly, the label was observed at the upper region of archenteron, and at some secondary mesenchyme cells in the blastocoel (Figs. 6H-J).

Effects of chemical treatment on apical plate formation

Neither depletion of *an1* blastomeres, nor formation of chimerical embryos drastically altered the size of animal plate (Tables 1, 2). Aiming at changing the size of animal

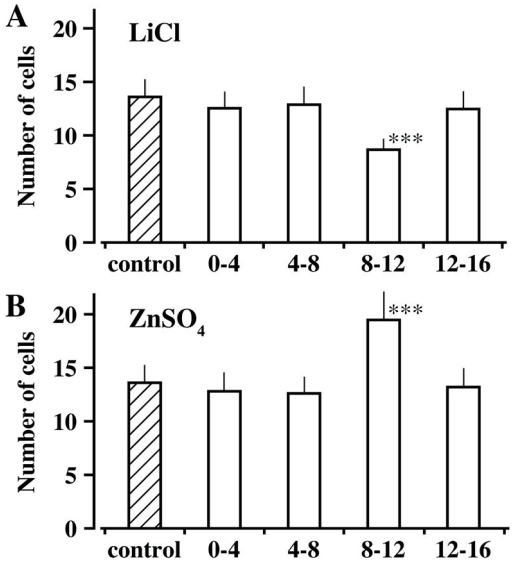

Figure 7. The number of cells in the animal plate of LiCl- or $ZnSO_4$-treated embryos

A; the number of cells in the animal plate of LiCl-treated embryos. **B**; the number of cells in the animal plate of $ZnSO_4$-treated embryos. Embryos were treated with LiCl or $ZnSO_4$ for every 4 hours. Abscissa; period of treatment. Ordinate; the number of cells in the animal plate. Attached vertical thin lines indicate S.D. ***: the difference between control embryos and treated embryos is statistically significant ($P < 0.001$). At each observation points, 15 embryos were examined.

plate more drastically, we tried to treate embryos with LiCl or $ZnSO_4$, which are thought to activate or inhibit Wnt signaling pathway (Poustka *et al*., 2007). As shown in Figure 7A, LiCl diminished the animal plate when it was applied to embryos during 8-12 hpf (from the swimming to mesenchyme blastula stage). As well, the effective period of $ZnSO_4$ treatment was 8-12 hpf (Fig. 7B).

Examples of chemically treated embryos are shown in Figure 8. LiCl treatment diminished the animal plate (Fig. 8B), while $ZnSO_4$ expanded the animal plate (Fig. 8C). Morphological characteristics of LiCl- or $ZnSO_4$-treated embryos are listed in Table 3. The animal plate of LiCl-treated embryo was significantly thinner than that of control embryo, and almost the same as that of the an_1-depleted embryos (Table 1). The thickness of animal plate in $ZnSO_4$-treated embryos was almost the same as that in control embryos; $ZnSO_4$ treatment expanded the size of animal plate, but did not cause further thickening of the animal plate. The thickness of lateral blastocoel wall in LiCl-treated or $ZnSO_4$-treated embryos was significantly thinner than that of control embryos. However, the difference was small. The length of apical tuft was significantly shorter in $ZnSO_4$-treated embryo, while the length of beating cilia was similar among control embryos, LiCl-treated embryos, and $ZnSO_4$-treated embryos.

LiCl and $ZnSO_4$ are potent reagents that alter the animal-vegetal polarity of sea urchin embryos. Therefore, treatment with these chemicals may cause the shifts of the ectoderm/endoderm boundary, affecting the formation of the animal plate. To examine this possibility, one of blastomeres of the 8-cell stage embryo was injected with a tracer dye, and the labeled embryos were observed at the end of gastrulation. In control embryos, the descendants of animal blastomere were distributed from the animal pole to the anal plate ectoderm (Figs. 8D-F). In the embryo treated with LiCl during 8-12 hpf, in which the vegetal blastomere had been labeled, the labeled descendants were distributed in the archenteron and the anal plate ectoderm (Figs. 8G-I). The distribution of labeled descendants in a $ZnSO_4$-treated embryo was the same as in control embryos (Fig. 8J-L). Thus, the position of the ectoderm/endoderm boundary was not altered under the treatment conditions used in this experiment.

Formation of apical ganglion in chemically treated embryos

In control embryos, about three serotonergic cells appeared at the early pluteus stage (30 hpf, Figs. 9A, B). They had begun to form a ganglion, which spanned about a half of the thickening region. In LiCl-treated embryos a smaller number of serotonergic cells appeared (Figs. 9C, D), and the width of apical ganglion was much smaller than in control embryos. On the contrary, a larger number of serotonergic cells appeared in $ZnSO_4$-treated embryos (Figs. 9E, F). The width of apical ganglion was about 1.5 times compared with that of control embryos.

At the mid-pluteus stage (48 hpf), the external morphology was indistinguishable between control larvae and $ZnSO_4$-treated larvae. In control larvae, 5-6 serotonergic cells formed the apical ganglion, spanning two antero-lateral arms (Figs. 9G, H). In the $ZnSO_4$-treated larvae, numerous serotonergic cells formed the apical ganglion (Figs. 9I, J). The width of such a ganglion was considerably larger than

Table 3. Morphological characteristics of the late-gastrula stage embryo treated with LiCl or $ZnSO_4$

	Number of animal plate cells	Thickness of animal plate (μm)	Thickness of lateral wall (μm)	Length of apical tuft (μm)	Length of cilia (μm)
Control	$12.6 \pm 1.3^{\S}$	21.3 ± 2.8	11.5 ± 0.9	58.8 ± 3.8	22.9 ± 2.0
LiCl¶	$1.3 \pm 1.7^{***}$	$13.6 \pm 1.7^{***}$	$10.3 \pm 1.5^{**}$	59.1 ± 3.9	22.9 ± 2.9
$ZnSO_4$¶	$18.8 \pm 2.2^{***}$	21.1 ± 2.3	$10.1 \pm 1.2^{**}$	$52.9 \pm 3.8^{***}$	21.9 ± 2.2

§: average and standard deviation were obtained by examining 15 embryos. ¶: embryos were treated with LiCl (30 mM) or $ZnSO_4$ (1mM) during 8-12 hpf. **: the difference in the averages between control embryos and manipulated embryos is statistically significant ($P < 0.01$). ***; $P < 0.001$.

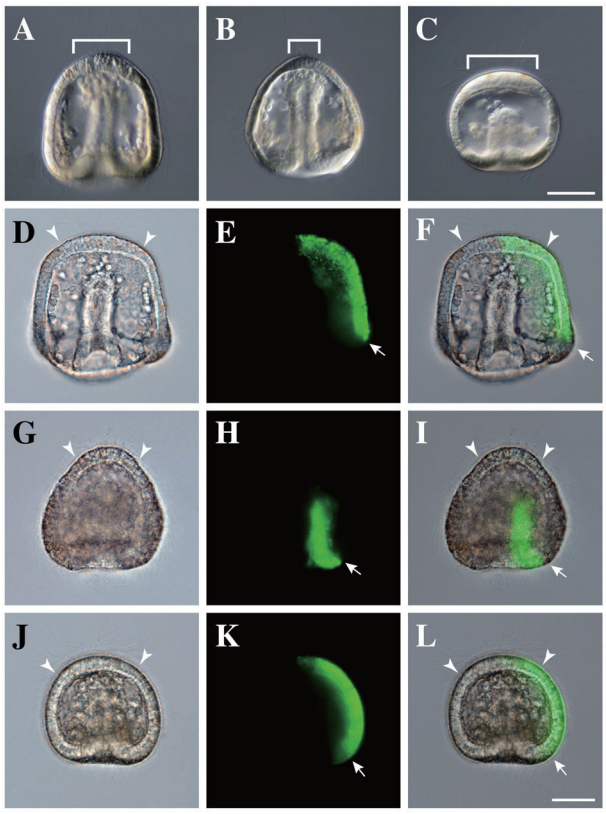

Figure 8. Animal plate formation in LiCl- or $ZnSO_4$-treated embryos

A; a control embryo. **B**; an embryo treated with LiCl from 8 to 12 hpf. **C**; an embryo treated with $ZnSO_4$ from 8 to 12 hpf. **A**-**C**; DIC images. Embryos were observed at 24 hpf. Brackets indicate the apical tuft forming area (animal plate). Small or large animal plate was formed in LiCl-treated or $ZnSO_4$-treated embryo, respectively. The scale bar in **C** is common to **A** and **B**, and indicates 50 μm. **D**-**F**; a control embryo injected with Lucifer Yellow into a vegetal blastomere at the 8-cell stage. **G**-**I**; a LiCl-treated embryo whose one vegtal blastomeres was labeled. **J**-**L**; a $ZnSO_4$-treated embryo whose one animal blastomeres was labeled. **D**, **G**, **J**; bright field images of control embryos, LiCl-treated embryos and $ZnSO_4$-treated embryos at 24 hpf. **E**, **H**, **K**; fluorescence images of **D**, **G** and **J**. **F**, **I**, **L**; merged images. Arrowheads indicate the boundary of thickened region. Arrows indicate the boundary between the animal hemisphere and vegetal hemisphere. Both chemically treated embryos show a delay in development, especially in the expansion of blastocoel. The scale bar in **L** is common to **D**-**K**, and indicates 50 μm.

that of control larvae.

Discussion

Findings in this study

Hörstadius (1973) reported a slight difference in the size of apical tuft forming area between the an_1- and an_2-blastulae.

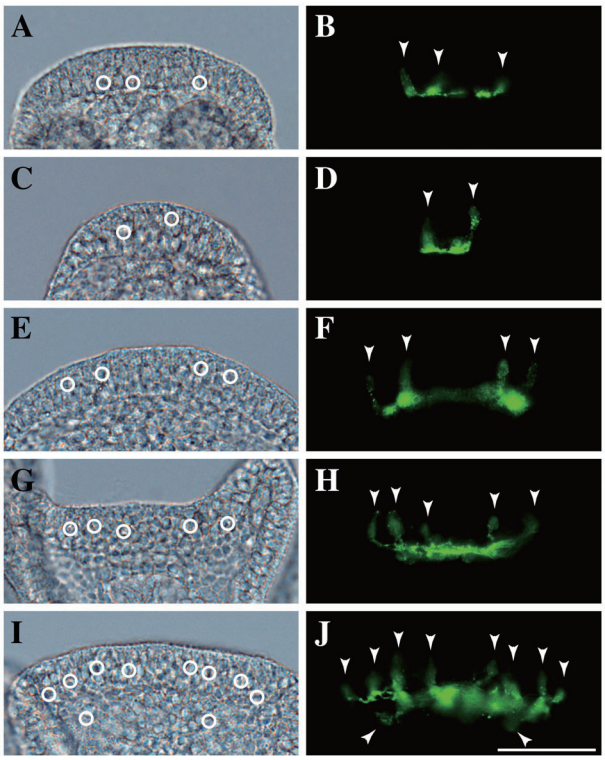

Figure 9. Formation of the apical ganglion in LiCl- or $ZnSO_4$-treated embryos

A, **B**; a control embryo at 30 hpf. **C**, **D**; a LiCl-treated embryo at 36 hpf. **E**, **F**; a $ZnSO_4$-treated embryo at 36 hpf. **G**, **H**; a control embryo at 48 hpf. **I**, **J**; a $ZnSO_4$-treated embryo at 48 hpf. **A**, **C**, **E**, **G**, **I**; bright field images. **B**, **D**, **F**, **H**, **J**; fluorescence images. Circles in bright field images indicate the positions of cell body of serotonergic cells. Arrowheads in fluorescence images indicate serotonergic cells. The scale bar in **J** is common to all figures, and indicates 50 μm.

Except his observation, however, no information is available about the difference in developmental potency between an_1 and an_2 tiers of blastomeres, because of the difficulty in isolating each tier of blastomeres. In this study, we successfully isolated an_1 and an_2 tiers from the 32-cell stage embryos, owing to comparatively large size of eggs, and a thin hyaline layer that surrounds the embryo. Further, we conducted some manipulations employing the isolated an_1 blastomeres to reveal their roles in the formation of animal plate and apical ganglion.

The notable findings are as follows. The thickening ability of cells forms a gradient from the animal pole toward the equator (Figs. 1, 2). The restriction signal begins to operate as early as the 16-cell stage (Fig. 2). The an_2-blastula forms a larger number of serotonergic cells than the an_1-blastula

(Fig. 3G). Serotonergic cells are preferentially distributed in thinner blastula wall region both in an_1- and an_2-blastulae (Fig. 3). Both animal plate thickening and differentiation of serotonergic cells depend on the number of an_1 blastomeres (Fig. 4, Table 1). The an_2 blastomeres or their descendants act as a barrier to the vegetal signal (Fig. 5, Table 2). Developmental potency of an_1 blastomeres is easily altered by environmental cues (Fig. 6).

Pulse treatment with chemicals gave us further information about the formation of apical organ. The size of animal plate is altered stage-specifically by LiCl or $ZnSO_4$ treatment (Figs. 7, 8, Table 3). The thickness of animal plate in embryos treated with $ZnSO_4$ does not exceed that in control embryos (Table 3). The width of apical ganglion closely correlates with the size of animal plate (Fig. 9).

A model for the apical organ formation

Based on the findings described above, we propose a model for the apical organ formation (Fig. 10A-D). This model contains following assumptions. (1) Cytoplasmic factors that thicken the ectodermal epithelium form a gradient from the animal pole toward the equator. (2) The thickening factors are inactivated by the vegetal signal. (3) A low concentration of thickening factors promotes the differentiation of serotonergic cells, while factors inhibit serotonergic cell differentiation at higher concentrations.

In the animal hemisphere, thickening factors form a concentration gradient from the animal pole to the equator (Fig. 10D, dotted line). During early cleavage stage, the signal emanated from the veg_1 tier of blastomeres restricts the activity of thickening factors especially at the an_2 tier (Fig. 10A). Therefore, the gradient of blastocoel wall thickness detected by separation of an_1 and an_2 tiers at the 32-cell stage does not necessarily show the original gradient in unfertilized eggs or eggs immediately after fertilization. Instead, the original gradient of thickening factors might be reflected in a gradual change in the thickness of blastocoel wall of delta-cadherin-injected embryos (Yaguchi *et al*., 2008).

At late cleavage stages, the vegetal signal is transmitted to the animal hemisphere, and further restricts the factors contained in an_1 tier of blastomeres (Figs. 10B, C). From the

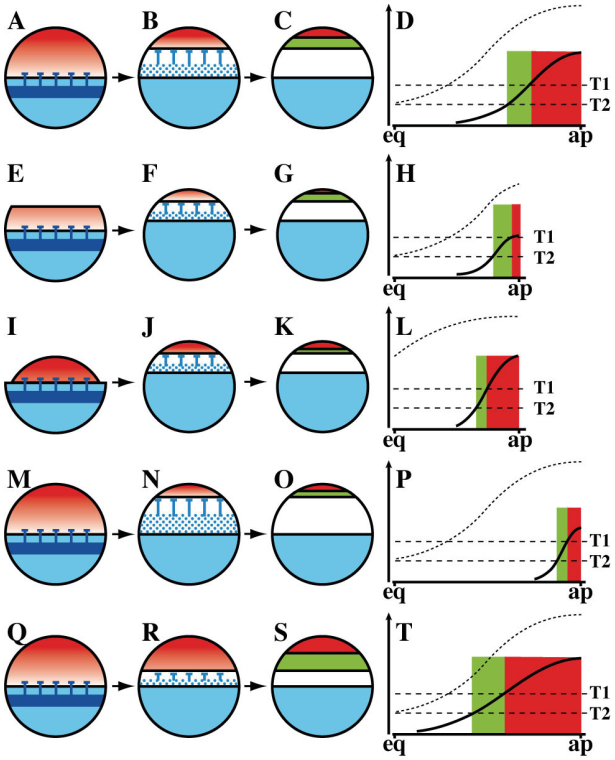

Figure 10. Diagrammatic representation of specification process of the thickening region and neurogenic area

A-**D**; specification process in normal embryo. **E**-**H**; an_1 tier-depleted embryo. **I**-**L**; an_2-depleted embryo. **M**-**P**; LiCl-treated embryos. **Q**-**T**; $ZnSO_4$-treated embryos. **A**, **E**, **I, M**, **Q**; at the 32-cell stage. The vegetal signal starts from the subequatorial region (dark blue). **B**, **F**, **J**, **N**, **R**; during late cleavage stages. **C**, **G**, **K**, **O**, **S**; at the mesenchyme blastula stage. **D**, **H**, **L**, **P**, **T**; presumed concentration gradients of thickening factors at the 32-cell stage and at the mesenchyme blastula stage are illustrated by dotted curve and thick curve, respectively. Abscissa; position along the animal-vegetal axis (arbitrary scaled). eq; equator. ap; animal pole. Ordinate; concentration of thickening factors. T1; threshold of thickening factors that causes the thickening of animal plate. T2; threshold of thickening factors necessary for the differentiation of serotonergic cells. For detail, see text.

swimming to mesenchyme blastula stage, the animal plate (expressed by red) is specified at the region where a large amount of thickening factors exists (Fig. 10D). Neurogenic area (expressed by light green) is specified beneath the animal plate, where a low concentration of thickening factors is present.

As shown in Figure 7, the sensitive period to LiCl or $ZnSO_4$ spans the swimming and mesenchyme blastula stages (8-12 hpf). Here, it should be reminded that the restriction process starts as early as the 16-cell stage. Therefore, such an

early vegetal signal is insensitive to the employed chemicals. The vegetal signal, which is designated as signal X in Yaguchi *et al*. (2008), must be composed of two different signaling systems. This coincides well with the finding that restriction of animal hemisphere progresses in two steps (Sasaki and Kominami, 2008).

From the late mesenchyme blastula stage, the animal plate region becomes thickened, owing to the activity of thickening factors. After the animal plate formation, a lower concentration of the factors would direct the differentiation of serotonergic cells. Simultaneously, neurogenic area is restricted toward the aboral side by Nodal signaling (Duboc *et al*., 2004: Yaguchi *et al*., 2006).

Apical organ formation in manipulated, or chemically treated embryos

In an_1-depleted embryos, the animal pole region contains only a low concentration of thickening factors (Fig. 10E). On receiving the vegetal signal, the activity of thickening factors is weakened (Fig. 10F), resulting in the loss of thickening area (Fig. 10G). Nevertheless, a low concentration of factors (Fig. 10H) promotes the differentiation of a small number of serotonergic cells near the animal pole (Figs. 4G, H).

In an_2-depleted embryos, the animal part initially contains a larger amount of thickening factors (Fig. 10I). The activity of such factors is also inactivated with the vegetal signal during late cleavage stages (Fig. 10J). By the mesenchyme blastula stage, the signal would reach the neighborhood of the animal pole (Fig. 10K), forming a steeper gradient of thickening factors (Fig. 10L). This leads to the narrowing of neurogenic area, resulting in the decrease of serotonergic cells (Fig. 5H).

LiCl treatment enhances the vegetal signal (Fig. 10N). As a result, the activity of thickening factors is inactivated except the animal pole region, forming a steeper gradient (Figs. 10O, P). This also causes the decrease in the number of serotonergic cells, in addition to the loss of thickened region.

As stated earlier, the restriction process would start as early as the 16-cell stage. The early phase of restriction process seems to be insensitive to $ZnSO_4$ treatment (Figs. 7B, 10Q). However, further restriction does not occur in the presence of $ZnSO_4$, and the activity of thickening factors remains in most part of the animal hemisphere (Fig. 10R). Accordingly, a gently-sloping concentration gradient is formed (Fig. 10T), resulting in the expansion of thickened region, and in increase in the number of serotonergic cells (Figs. 9I, J).

Size and thickness of the animal plate

At the late gastrula stage, animal plate cells are arranged in about 12 cells width and 3-5 cells depth (Cameron *et al*., 1993: Sasaki and Kominami, 2008). When an_1 blastomeres had been removed at 32-cell stage, the animal plate became thinner as the number of removed cells was increased (Table 1). Nonetheless, the size of animal plate remained rather constant. In extreme case, animal plate cells were formed, in spite of the lack of an_1 blastomeres (Fig. 4D). Besides the action of thickening factors, some unknown mechanisms would determine the size of animal plate in manipulated embryos. Likewise, we could not find intensive correlation between the thickness of animal plate and the length of apical tuft (Tables 1-3). The amount of ankAT-1, a transcription factor that contains ankyrin repeats, is likely to regulate the length of long apical tuft cilia (Yaguchi *et al*., 2010). However, it is unknown whether ankAT-1 is involved in the determination of the thickness of animal plate.

Entities of the presumed thickening factors

Yaguchi *et al*. (2008) reported the expression pattern and functions of FoxQ2, a member of forkhead gene family. FoxQ2 begins to be expressed in the whole animal hemisphere at the 32-cell stage. As the progress of cleavages, FoxQ2-expressing region becomes restricted toward the animal pole side. At the mesenchyme blastula stage, FoxQ2 is specifically expressed in the animal plate. This expression pattern coincides well with the restriction process of animal hemisphere revealed by manipulative methods (Sasaki and Kominami, 2008). However, the animal plate does thicken even if the embryo is injected with FoxQ2 morpholino. Further, over-expression of FoxQ2 blocks the thickening of animal plate (Yaguchi *et al*., 2008). Thus, FoxQ2 is not the entity of presumed thickening factors. FEZ, a zinc finger

protein, is expressed specifically in the animal plate (Yaguchi *et al*., 2011). This transcription factor is also irrelevant to the presumed thickening factors, since FEZ is a downstream gene of FoxQ2, and is expressed much later than the stage of animal plate specification.

It is natural to suppose that serotonergic cells are derived from the an_1-descendants near the animal pole. However, the number of differentiated serotonergic cells was much larger in an_2-blastulae than in an_1-blastulae (Fig. 3). Some an_1-blastulae did not serotonergic cells at all. Further, the number of serotonergic cells that form the apical ganglion seemes to be smaller in an_2-depleted embryos than in an_1-depleted embryos (Figs. 4I, 5H). The possibility cannot be excluded that some of serotonergic cells are derived from an_2-tier of blastomeres, and they move towards the animal pole. The data and model presented in this study would serve for unveiling the events occurring at cleavage stages, and for analyzing the upstream factors of the genes involved in the specification and formation of apical organ.

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