Determination of first cleavage plane: the relationships between the orientation of the mitotic apparatus for first cleavage and the position of meiotic division-related structures in starfish eggs

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Abstract

In order to understand when the orientation of the first cleavage plane is fixed along the animal–vegetal axis in starfish eggs, the behavior of the sperm aster was examined by indirect immunofluorescence staining. After duplication, the sperm aster organizes the mitotic apparatus for first cleavage perpendicular to the cleavage plane. The sperm aster located in the egg periphery just after fertilization and moved to the site close to the animal pole rather than the egg center by meiosis II. At early metaphase II, duplication of the sperm aster was detected but the axis of the resultant sperm diaster randomly pointed. Subsequently, its axis had already turned perpendicular to the animal–vegetal axis before pronucleus fusion. These results indicate that the orientation processes of the sperm diaster consist of positioning before its duplication and successive determining its azimuth. Furthermore, the azimuth and position of the mitotic apparatus for first cleavage did not change by shifting or eliminating the meiotic division-related structures such as the germinal vesicle, meiotic spindle, and female pronucleus by micromanipulation. These results show that none of them determines the first cleavage plane. Therefore, we discuss the pointing mechanism of the first cleavage plane without the influence of these meiotic division-related structures.

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Introduction

In many animal species, the orientation of cell divisions determines the overall organization of the embryo and the relative positioning of embryonic cells or tissues in the embryo. Furthermore, some divisions in early embryos are “determinative,” producing daughter cells with different developmental fates (Roegiers et al., 1999). A specific orientation of the cleavage plane can differentially partition localized cellular components into the daughter cells and, on the other hand, can equally partition such localized components into them. Especially, the first cleavage plane is through the animal–vegetal axis in many animal species including the starfish and sea urchin, and divides the egg in equal blastomeres.

The cleavage furrow forms equidistantly between the two poles of the mitotic apparatus (MA) (Hiramoto, 1956; Rappaport, 1971). Therefore, the pattern of cleavage depends upon the controlling mechanisms of the position and azimuth of MA. In this way, the controlling mechanism of the MA orientation is important for cell cleavage. In recent years, a considerable number of studies have been conducted on the controlling mechanism of the orientation of MA in the early embryos of C. elegans, neuroblasts of Drosophila, epithelial cells (Busson et al., 1998; Dujardin and Vallee, 2002; Skop and White, 1998). However, the mechanism for orienting the cleavage plane through the...
animal–vegetal axis and inducing equal division such as early cleavages of the starfish and sea urchin eggs has not yet been investigated.

In starfish eggs, the plane for first mitotic cleavage is through the animal–vegetal axis. In immature oocytes, the germinal vesicle does not locate the geometric center of the egg, but is attached to the cortex, which is historically defined as the animal pole (Schroeder, 1985). Some factors responsible for archeronten formation are concentrated in the region around the vegetal pole at this stage (Kuraishi and Osanai, 1994). Thus, starfish immature oocytes express an axis of polarity along the line of the animal and vegetal poles. The oocytes are arrested at early prophase of the first meiotic division. Upon reinitiating meiosis that is induced by 1-methyladenine (1-MeAd), the germinal vesicle breaks down. One pole of the meiotic spindle is attached to the animal pole cortex, where polar bodies eventually form (Hamaguchi, 2001; Kominami, 1983; Shirai and Kanatani, 1980). After the second meiotic division, the female pronucleus migrates toward the male pronucleus and fuses with it (Hirai et al., 1981). Thereafter, the mitotic apparatus for first cleavage (the first MA) forms perpendicular to the animal–vegetal axis.

In this study, we quantified the distance between the sperm aster and the animal pole by indirect immunofluorescence from meiosis to formation of the first MA. Especially, in order to reveal when and how the axis of the sperm diaster, the line between the two centers of the sperm diaster, points appropriately for first cleavage, we examined the azimuth of the sperm diaster just after its duplication. Soon after sperm entrance, the sperm aster became located in the egg periphery. At meiosis II, the sperm aster moved close to the animal pole. During early metaphase II, just after duplication of the sperm aster, its axis did not yet point appropriately. After the second polar body formation, it pointed appropriately.

Furthermore, we examined whether or not the position of these meiotic division-related structures, the germinal vesicle, the spindle, and the female pronucleus, determines the orientation of the first MA because the position of these meiotic division-related structures corresponds to the site where the first cleavage plane is. Contrary to our expectation, the orientation of the first MA did not change in spite of shifting or eliminating these meiotic division-related structures.

**Materials and methods**

**Culture**

Oocytes were obtained from the ovaries of the starfish, Asterina pectinifera, and washed in Ca-free sea water (Ca-free Jamarin U, Jamarin Lab., Osaka, Japan) to remove the follicle cells (Saiki and Hamaguchi, 1998). Sperm was collected as “dry sperm,” kept at 4°C in a refrigerator, and diluted just before use. Oocytes were treated for 20 min with 2 μM 1-MeAd in order to resume meiosis (Kanatani, 1969). Maturing oocytes were inseminated 40 min after the 1-MeAd application by adding a sperm suspension which was freshly made of dry sperm. Fertilized eggs were deprived of the fertilization envelope by treating them for 3 min with 1% thioglycolate in ASW shortly after insemination and washed three times with Ca-free sea water (Maruyama et al., 1986). All experiments were carried out at 19–21°C.

**Indirect immunofluorescence**

Indirect immunofluorescence microscopy was carried out using the anti-tubulin antibody according to Oka et al. (1994) and Hamaguchi (1998) with some modifications. It is summarized below. The eggs were extracted for 60 min with an MT-stabilizing solution (5 mM EGTA, 20 mM Pipes, 2 mM MgCl₂, 1 M glycerol, the pH was adjusted to 7.0 by KOH) supplemented with surfactants of 1% Nonidet P-40 and 0.2% CHAPS. The extracted eggs were attached to a 0.01% polylysine-coated coverslip. These eggs were fixed for 20 min with 0.3 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and then for 20 min with 1.8% formaldehyde in the MT-stabilizing solution without surfactants. They were stained for 60 min at 37°C with the monoclonal anti-tubulin antibody (DM1A, Amersham, Buckinghamshire, England) as the first antibody and, after rinsing, for 60 min at 37°C with FITC-conjugated anti-mouse IgG antibodies (Kirkegaard and Perry Lab. Inc., Gaithersburg, MD) as the secondary antibodies. The first antibody and the anti-mouse antibodies were used after diluting 1000 and 100 times, respectively. Chromosomes were also fluorescent after staining with PI (propidium iodide) at 0.1 μg/ml for 30 min.

**Micromanipulation**

The micromanipulation was carried out using a micromanipulator and an injector (MO-102R and IM-4, Narishige Sci. Inst. Lab., Tokyo, Japan) as follows (Hamaguchi, 2001). Micropipettes were made with a needle puller from a glass capillary with a 1-mm outer diameter. The pipette was sometimes filled with paraffin oil for marking. The oocytes were placed in a trough at a thickness of 100 μm for manipulation and observation.

**Shifting the germinal vesicle contents**

In order to enucleate the germinal vesicle, the tip of a micropipette was inserted into the cell membrane where the germinal vesicle was attached and then moved in the direction opposite to the center of the oocyte (Hirai et al., 1971). The wound made by manipulation was defined as a marker of the animal pole. Thereafter, the contents of the germinal vesicle from another oocyte were injected into the
cytoplasm which was distant by 90° from the animal pole instead of transplanting another germinal vesicle. At the same time, an oil drop was injected and defined as the marker of the site where the contents of the germinal vesicle were injected. These oocytes were defined as the germinal vesicle-shifted eggs.

**Shifting the first meiotic spindle**

Shifting the first meiotic spindle was carried out according to Hamaguchi (2001). An oil drop was injected as the marker of the animal pole between the fertilization envelope and the cell membrane where the pole of the meiotic spindle was attached. The tip of a micropipette was inserted into the cytoplasm which was distant by 90° from the animal pole. The cortex, where the polar bodies formed, was defined as the position to which the meiotic spindle was shifted. Those oocytes were defined as meiotic spindle-shifted eggs.

**Eliminating the female pronucleus**

The tip of a micropipette was inserted into the cytoplasm beneath the second polar body before the female pronucleus started to migrate, and then the female pronucleus was aspirated from the starfish eggs (Hirai et al., 1971). These eggs were defined as the female pronucleus-eliminated eggs.

**Microscopy and image analysis**

Living oocytes and eggs were observed using a differential interference contrast (DIC) and polarization microscope (Optiphot, Nikon, Tokyo, Japan). The image was taken by video-enhanced microscopy, which was carried out using a CCD camera (FIT CCD WLV730, Panasonic, Tokyo, Japan) and a real-time image processor (ARGUS-20, Hamamatsu, Japan). The centers of the asters in the living oocytes and eggs were manually determined in the polarization micrographs.

Three-dimensional images of fixed eggs were collected using a laser scanning confocal microscope (Zeiss LSM510, Carl Zeiss MicroImaging, Inc.) with a 40× objective. Critical illumination was used giving a spatial resolution of about 0.22 μm. Optical sectioning was carried out at the interval of 1.43 μm in depth. Some figures in the Results section were generated by projecting a few optical sections. To determine the position of the sperm asters, the images were processed with IPLab (Solution Systems, Chiba, Japan) as follows. To discriminate the fluorescence image of sperm asters from the background fluorescence of the cytoplasm, the maximal fluorescence intensity of the cytoplasm in the eggs, where the regions of the sperm aster and the meiotic spindle were excluded, was subtracted from each two-dimensional image and the image of the sperm aster was separated from the resultant image. The three-dimensional weighted center of the fluorescence intensity of the sperm aster was then calculated from all of the two-dimensional images. The position of the animal pole in the living or fixed oocytes and eggs was determined as follows. In case of the meiotic spindle existing in them, the cortex, where the pole of the meiotic spindle was attached, was defined as the animal pole.

The azimuth of the presumptive plane for first cleavage was defined as follows. A plane through the center of the first MA and perpendicular to its axis was determined as the presumptive cleavage plane (Fig. 1a). The angle between the line from the animal pole to the center of the first MA and the presumptive cleavage plane was defined as \( \theta_{\text{AP}} \). When the meiotic division-related structures were shifted, the angle between the meiotic division-related structures and the presumptive cleavage plane was defined as \( \theta_{\text{EQ}} \). The values of \( \theta_{\text{AP}} \) and \( \theta_{\text{EQ}} \) range from 0° to 90°.

We measured the position of the first MA as follows. The distance between the center of the first MA and the animal pole membrane was determined as \( l_{\text{AV1}} \), and the distance between the center of the first MA and the membrane opposite to the animal pole was determined as \( l_{\text{AV2}} \) (Fig. 1b). To normalize the position of the first MA, \( R_{\text{AV}} \), a relative position from the egg center, was calculated using the Eq. (1).

\[
R_{\text{AV}} = \left( l_{\text{AV2}} - l_{\text{AV1}} \right) / \left( l_{\text{AV2}} + l_{\text{AV1}} \right) \times 100
\]

\( R_{\text{AV}} \) ranges from −100 to 100. If \( R_{\text{AV}} \) is more than 0, then the first MA is off the middle and close to the animal pole.

![Fig. 1. Schematic representation of measuring the azimuth and position of the first MA.](image-url)
on the line that is through the center of the first MA from the animal pole to the opposite membrane. If $R_{av}$ is 0, the first MA is in the middle, although the middle of the line does not always correspond to the geometric center of the egg. When the meiotic division-related structures were shifted, $R_{eq}$ was calculated in a similar manner, in reference to the marker of the meiotic division-related structures.

Fig. 2. The morphological and positional changes of the sperm aster from meiosis I to MA formation at first cleavage. Microtubules and chromosomes were visualized by confocal microscopy. Figures were generated by projecting some optical sections. Green and red are microtubules and chromosomes, respectively. In order to show the outline of eggs, the intensity of the red image of PI was enhanced. The time after application of the 1-MeAd is indicated at the upper left of each picture. 60 min. Metaphase I. The meiotic spindle (MS) was formed at the animal pole. The sperm aster was located in the egg periphery and the astral rays were short. The sperm nucleus (M) was located nearby the sperm aster. 75 min. Anaphase I. The sperm astral rays elongated. 90 min. Early metaphase II. The polar body (PB) formed. The sperm astral rays shortened. The duplicated sperm asters (arrows) were close to the animal pole and near the sperm pronucleus (M). 105 min. Anaphase II. The sperm aster was enlarged. 120 min. Pronucleus fusion. The sperm diaster was observed in the vicinity of the sperm nucleus (arrows). 135 min. The formation of the first MA. Scale bar, 50 μm.
Results

Sperm aster development and positioning

Fixed eggs were fluorescently stained and observed using a confocal microscope (Fig. 2). At 60 min after application of 1-MeAd, the metaphase the meiotic spindle was observed just beneath the animal pole cortex (Fig. 2, 60 min). The sperm aster had a small size and was in the egg periphery. At 75 min, the oocytes were at anaphase I. The distance between the sperm nucleus and the sperm aster increased. A strong fluorescence intensity was observed between the aster and the sperm nucleus. The astral rays asymmetrically elongated and showed a comet-like shape. At 90 min, the oocytes were at early metaphase II and the sperm aster became small. The distance between the sperm aster and the sperm nucleus decreased. The sperm diaster was observed in some eggs (2/10). At 105 min, the second meiotic division occurred. The condensed sperm chromosomes were observed in a small number of eggs (3/13). The male pronucleus formed in the cytoplasm beneath the animal pole. At 120 min, the condensed sperm chromosomes were observed in some eggs (2/10). At 105 min, the sperm aster was at the head of the sperm aster, which asymmetrically developed and appeared to be suppressed at the animal pole. At 120 min, the female pronucleus formed in the cytoplasm beneath the animal pole. The condensed sperm chromosomes were observed in small number of eggs (3/13). The male pronucleus formed in some eggs (3/13). The pronucleus fusion occurred in the other eggs (7/13). At 135 min, the first MA at metaphase formed. Table 1 summarizes the distance between the animal pole and the sperm asters or spindle poles. At 60 min and 75 min, the distance between the animal pole and the sperm aster was about 100 μm. At 90 min, the distance dramatically decreased to about 60 μm. From 90 min to 135 min, the sperm aster and the mitotic apparatus for first cleavage kept a consistent distance from the animal pole.

The azimuth of the sperm diaster or the first MA (θ_AP)

θ_AP was quantified with the weighted center of the fluorescence intensity of the sperm asters or the asters of the first MA at 90, 120, and 135 min after 1-MeAd application (Table 1 and Fig. 3). Since a small number of sperm asters was duplicated at 90 min, we obtained 6 examples from the many examined eggs. At 90 min, θ_AP was 54.0° as shown in Table 1, which was significantly different from θ_AP at 135 min (P < 0.001). This result shows that the axis of the sperm diaster did not yet point perpendicular to the animal–vegetal axis. Since the sperm asters did not appear as two asters at 105 min, we could not quantify θ_AP. At 120 min, θ_AP became 13.8°, which means that the axis of the sperm diaster already pointed perpendicular to the animal–vegetal axis. Moreover, we investigated whether or not the azimuth of the sperm diaster is related to the pronuclear fusion. Although pronuclear fusion occurred in some eggs, but did not occur in the other eggs, there is no significant difference in θ_AP between the two types of eggs (Table 1). At 135 min, the axis of the first MA pointed perpendicular to the animal–vegetal axis. Consequently, in Fig. 4, the process of sperm aster orientation is schematically summarized. The sperm aster was located near the point where the sperm penetrated just after the fertilization. Thereafter, the sperm aster began to migrate and was close to the animal pole during meiosis II. After this positioning, the sperm diaster duplicated, but its axis randomly pointed. However, its axis was already pointed appropriately for first cleavage before pronuclear fusion.

Table 1

<table>
<thead>
<tr>
<th>Time after 1-MeAd application (min)</th>
<th>Distance between the animal pole and the sperm aster or mitotic apparatus (μm) (n)</th>
<th>Distance between θ_AP (°) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>94.6 ± 29.7 (10)</td>
<td>6.5 ± 3.0 (10)</td>
</tr>
<tr>
<td>75</td>
<td>100.5 ± 29.6 (10)</td>
<td>12.2 ± 2.9 (9)</td>
</tr>
<tr>
<td>90</td>
<td>54.0 ± 15.4 (10)</td>
<td>9.3 ± 1.9 (9)</td>
</tr>
<tr>
<td>105</td>
<td>71.3 ± 9.0 (8)</td>
<td>54.0 ± 25.6 (6)</td>
</tr>
<tr>
<td>120</td>
<td>42.4 ± 6.3 (12)</td>
<td>13.8 ± 10.0 (12)</td>
</tr>
<tr>
<td>135</td>
<td>58.7 ± 3.1 (12)</td>
<td>14.5 ± 12.0° (5)</td>
</tr>
</tbody>
</table>

θ_AP is the angle between the animal–vegetal axis and the plane perpendicular to the azimuth of the sperm diaster or the first MA. (n): Egg number.

a θ_AP before pronuclear fusion.
b θ_AP during pronuclear fusion.
The orientation of the first MA in normal eggs and control eggs

The meiotic division-related structures such as the germinal vesicle, the meiotic spindle, and the female pronucleus are the markers of the animal pole during meiosis and the first cleavage plane is through the animal pole. Therefore, it is reasonable to hypothesize that the meiotic division-related structures would be involved in the orientation of the first MA. We examined whether or not the orientation of the first MA was affected by shifting or eliminating the meiotic division-related structures.

First of all, the orientation of the first MA in living eggs was quantified without shifting the meiotic division-related structures or compressing the eggs. These eggs were defined as normal eggs (Fig. 5). In the normal eggs, the germinal vesicle breakdown, the meiotic divisions, and the female pronucleus formation occur at the animal pole. The angle between the animal–vegetal axis and the plane perpendicular to the azimuth of the first MA, $\theta_{\text{AP}}$, was $11.5^\circ$, which indicates that the presumptive plane was through the animal pole (Table 2 and Fig. 6 normal). There is no significant difference between this azimuth and $\theta_{\text{AP}}$ in fixed eggs. The distance between the animal pole and the first MA, $l_{AV1}$, was 61.1 $\mu$m, as shown in Table 3. There is no significant difference between the normal eggs and the fixed eggs. $R_{AV}$ of the normal eggs of 16.3% indicates that the first MA was not at the egg center, but close to the animal pole (Table 3). These results show that the presumptive cleavage plane was through the animal pole and the first MA was close to the animal pole.

In order to measure the orientation of the first MA without the difficulty of the three-dimensional measurement of position and azimuth, the eggs were compressed to locate the first MA, the animal pole and the meiotic division-related structures in the same focal plane. These eggs were defined as the control eggs. At first, the orientation of the first MA was measured without manipulating the meiotic division-related structures to estimate the effect of compressing. $\theta_{\text{AP}}$ of the control eggs of $20.8^\circ$ indicates that the presumptive cleavage plane was through the animal pole (Table 2 and Fig. 6 control). Although $\theta_{\text{AP}}$ slightly increased, there is no significant difference between the control eggs and the normal eggs. The result indicates that this compressing did not seriously affect the azimuth of the first MA. Therefore, in order to assess the effect of the shifting or eliminating the meiotic division-related structures, the manipulated eggs were compared to the control eggs.

In addition, $R_{AV}$ of the control eggs of 26.3% indicates that the first MA was closer to the animal pole than the normal eggs (Table 3). However, the average of $l_{AV1}$ in the control eggs was 67.1 $\mu$m, which is slightly significantly greater than the average of $l_{AV1}$ in the normal eggs. On the other hand, $l_{AV2}$ in the control was significantly greater than...
that in the normal eggs. In other words, although both $I_{N1}$ and $I_{N2}$ of the control eggs were greater than those of the normal eggs, $I_{N1}$ did not increase so greatly as $I_{N2}$ in the control eggs. These results indicate that the distance between the animal pole and the first MA tends to maintain a consistent length in spite of the increase in the egg diameter.

The first MA orientation of the germinal vesicle-shifted eggs

It has been reported that the oocytes do not mature nor cleave when there is no content of the germinal vesicle in the enucleated oocytes (Yamada and Hirai, 1984). Therefore, in the germinal vesicle-shifted eggs, after enucleation, the contents of the germinal vesicle were injected into the cytoplasm which was distant by 90° from the animal pole (Figs. 7a–c). In these eggs, although neither the polar body formation nor pronuclear fusion occurred, the first MA formed 120 min after the 1-MeAd application when the germinal vesicle would be contained in the germinal vesicle-shifted eggs (Fig. 7d).

We examined whether the presumptive cleavage plane was through the animal pole or through the cytoplasm where the contents of the germinal vesicle were injected. The averages of $\theta_{AP}$ and $\theta_{EQ}$ of the germinal vesicle-shifted eggs were 32.2° and 55.9°, respectively (Table 2 and Fig. 6 GV). There is no significant difference between $\theta_{AP}$ of the control eggs and the germinal vesicle-shifted eggs. On the other hand, there is a significant difference between $\theta_{AP}$ of the control eggs and $\theta_{EQ}$ of the germinal vesicle-shifted eggs as shown in Fig. 6. These results show that the presumptive cleavage plane was through the animal pole, but was not through the cytoplasm where the contents of the germinal vesicle were injected.

$R_{AP}$ of the germinal vesicle-shifted eggs was 10.5% and is significantly different from $R = 0$ (Table 3). On the other hand, $R_{EQ}$ of the germinal vesicle-shifted eggs was 4.0% (Table 3), and is not significantly different from $R = 0$. It is found from the result that the first MA was close to the animal pole, but not close to the cytoplasm where the contents of the germinal vesicle were injected.

The first MA orientation of the meiotic spindle-shifted eggs

The first meiotic spindle formed beneath the animal pole after the germinal vesicle breaks down (Fig. 8a). This meiotic spindle was shifted to the cortex which was distant by 90° from the animal pole. The first polar body and then the second one formed at the cortex where the meiotic spindle was shifted (Figs. 8b–c), which shows that the whole of the meiotic spindle including two centrosomes at the poles was shifted. The female pronucleus formed in the cytoplasm at the site where the meiotic spindle was shifted and fused with the male pronucleus. The first MA then formed (Fig. 8d).

$\theta_{AP}$ and $\theta_{EQ}$ of the meiotic spindle-shifted eggs were 30.7° and 57.4° (Table 2 and Fig. 6 MS), respectively, and showed that the presumptive cleavage plane was through the animal pole, but was not through the cortex where the meiotic spindle was shifted. Furthermore, $R_{AP}$ and $R_{EQ}$ of the meiotic spindle-shifted eggs of 18.9% and 0.5% (Table 3), respectively, show that the first MA was close to the animal pole, but was not close to the site where the meiotic spindle was shifted.

The first MA orientation of the female pronucleus-eliminated eggs

The germinal vesicle breakdown, the meiotic divisions, and polar body formation occur at the animal pole.
Thereafter, the female pronucleus forms beneath the animal pole. In this experiment, the female pronucleus was eliminated before the female pronucleus started to move to the male pronucleus (Figs. 9a–c). Therefore, the pronuclear fusion did not occur in these eggs and the first MA formed without the female pronucleus. The AP of the female pronucleus-eliminated eggs of 21.9 ± 8.8 shows that the presumptive cleavage plane was through the animal pole in spite of the elimination of the female pronucleus. The AV of the female pronucleus-eliminated eggs of 19.4% shows that the first MA was close to the animal pole in spite of the elimination of the female pronucleus. Consequently, the first MA in the female pronucleus-eliminated eggs oriented as normally as in the control eggs (Fig. 6 and Tables 2 and 3FP).

Discussion

Sperm aster positioning

In this study, between 75 min and 90 min after 1-MeAd application, the distance between the animal pole and the sperm aster dramatically changed from 100.5 μm to 64.3 μm in 15 min or less and then the distance did not change after 90 min after 1-MeAd application, which indicates that sperm aster positioning is almost over at 90 min. The sperm aster developed and showed an asymmetrical figure at 75 min. It has also been reported that the size of the sperm aster was affected by the cytoplasmic changes (Hirai et al., 1981). Therefore, the elongation of their astral rays would be involved in this migration. However, their astral rays do not appear to interact with the cortex at the end of this migration. The distance of ca. 100 μm between the vegetal pole and the sperm aster, which was appropriately positioned, would be too long for the astral rays to reach the cortex. Moreover, the astral rays would not interact with the animal cortex because it has been suggested that the development of the sperm astral rays was suppressed at the region of the animal pole during meiosis (Stephano and Gould, 2000). In the sea urchin egg, the Colcemid-UV method revealed that the sperm aster moves without interactions between the cortex and its astral rays (Hamaguchi and Hiramoto, 1986). It is quite likely that the same mechanism is involved during sperm aster movement in starfish eggs. However, there is a discrepancy; in starfish and normal sea urchin, the moving direction of the sperm aster was coincident with the direction of the shorter microtubules and, on the other hand, the direction was coincident with the direction of the longer microtubules in the sea urchin based on the Colcemid-UV method (Hamaguchi and Hiramoto, 1986). There would be additional mechanisms which control the moving direction of the sperm aster in normal starfish and sea urchin eggs.

From 90 min to 135 min after 1-MeAd application, the sperm aster was closer to the animal pole than to the vegetal pole. The distance between the animal pole and the sperm aster of the eggs fixed at 90 min was coincident with that of the normal egg at the first MA formation (Table 1, 90 min and Table 3), suggesting that the sperm aster appropriately

Table 3

<table>
<thead>
<tr>
<th></th>
<th>Distance with respect to the animal pole</th>
<th>Distance with respect to the shifted site</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( l_{AV1} (\text{μm}) )</td>
<td>( l_{AV2} (\text{μm}) )</td>
<td>( R_{AV} (%) )</td>
</tr>
<tr>
<td>Normal eggs</td>
<td>61.1 ± 2.9</td>
<td>85.0 ± 5.8</td>
<td>16.3 ± 4.2*</td>
</tr>
<tr>
<td>Control eggs</td>
<td>67.1 ± 11.7</td>
<td>114.3 ± 10.6</td>
<td>26.3 ± 8.3*</td>
</tr>
<tr>
<td>Germinal vesicle-shifted eggs</td>
<td>96.1 ± 17.7</td>
<td>119.3 ± 22.8</td>
<td>10.5 ± 15.4*</td>
</tr>
<tr>
<td>Meiotic spindle-shifted eggs</td>
<td>75.4 ± 9.6</td>
<td>111.0 ± 14.0</td>
<td>18.9 ± 10.6*</td>
</tr>
<tr>
<td>Female pronucleus-eliminated eggs</td>
<td>71.3 ± 10.9</td>
<td>118.7 ± 13.3</td>
<td>19.4 ± 6.5*</td>
</tr>
</tbody>
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*\( R \) which was significantly different from \( R = 0 \).
positions by meiosis II. The sperm aster was off the geometrical center of the eggs and kept a consistent distance from the animal pole (Table 1, 90–135 min). Moreover, under compression, the change in this distance was less than the change in the distance between the sperm aster and the side opposite to the animal pole (Table 3). These results indicate that there exists a mechanism, which maintains an appropriate distance between the sperm aster and the animal pole in starfish eggs.

**Pointing process of the axis of the sperm diaster for first cleavage**

The axis of the sperm diaster was appropriately pointed for first cleavage by pronuclear fusion (Fig. 3). This result is consistent with the results in the sea urchin and frog (Schatten, 1981; Stewart-Savage and Grey, 1982). There exist two possible processes of the axis of the sperm diaster pointing. One possibility is that the axis of the diaster already points appropriately during sperm aster separation. The other is that the axis of the sperm diaster does not point just after the sperm aster separation, and then the sperm diaster changes its relative position to make its axis point perpendicular to the animal pole. Because the separated sperm asters were observed at prophase II, but its axis did not appropriately point (Fig. 3, 90 min), it is likely that the latter possibility is correct in starfish eggs. In addition, the sperm aster was already positioned by meiosis II. Thus, it is suggested that the pointing mechanism is temporally independent of the positioning mechanism (Fig. 4).

**Effects of the position of meiotic division-related structures on appropriate azimuth of the first MA**

It has been suggested that the midbody position of the former division determines the azimuth of MA for the next cleavage. It was reported that the axis of MA for the second cleavage was through the midbody which was formed during the first cleavage in *C. elegans* (Skop and White, 1998). Furthermore, the cleavage plane for the first division was through the midbody which formed at the meiotic division in mouse (Plusa et al., 2002). However, in this study, the presumptive cleavage plane was not through the meiotic midbody as shown in the meiotic spindle-shifted eggs but was through the animal pole (Fig. 6 MS). In the germinal vesicle-shifted egg, which has no meiotic midbody, the presumptive cleavage plane was also through the animal pole (Fig. 6 GV). Thus, the possibility that the midbody at the meiotic division determines the azimuth of the first MA was denied in the starfish eggs.

The female pronucleus forms beneath the animal pole and migrates to the sperm asters. Thus, the trajectory of the female pronucleus is parallel to the animal–vegetal axis; i.e., the plane of first cleavage. It has been reported that this trajectory determines the azimuth of the first MA in *Urechis caupo* (Rouvière et al., 1994). In the female pronucleus-
eliminated eggs, however, the presumptive cleavage plane was through the animal pole (Fig. 6 FP). In the germinal vesicle-shifted eggs, female pronucleus migration could not occur because the female pronucleus did not form as a result of the germinal vesicle-elimination or the germinal vesicle-content injection and, in the meiotic spindle-shifted eggs, the trajectory of the female pronucleus was perpendicular to the animal–vegetal axis. Nevertheless, in both of these eggs, the presumptive cleavage plane was through the animal pole (Fig. 6 GV, MS). These results show that the trajectory of the female pronucleus does not determine the azimuth of the first MA.

The controlling mechanism of the pointing process of the sperm diaster

It is possible to propose two hypothetical models that explain the controlling mechanism of the pointing process of the sperm aster in the starfish egg from our results in this study. In both hypotheses, the cue for the pointing process is independent of the meiotic division-related structures and the first MA accomplishes the pointing process before pronuclear fusion. Assuming that the sperm astral rays interact with the cortex at the animal pole, the interaction would occur after meiosis during the stage after the elongation of the rays of the sperm aster. As we mentioned in the Results section, the elongation of the sperm astral rays appeared to be suppressed at meiosis II, which may result in the fact that the axis of the sperm diaster did not appropriately point at meiosis. The axis of the sperm diaster appropriately points after the elongation of the astral rays, which would be induced by the suppression of MAPK activity because it is suggested that MAPK suppresses sperm aster development at the animal pole until the end of meiosis II in the starfish egg (Stephano and Gould, 2000).

First, we propose the cortical model in which certain factors are located at the animal pole (Fig. 10a). When the rays of the sperm diaster reach the cortical factors, which are located at the animal pole, the sperm diaster is attracted toward the animal pole by interaction between the factor and the astral rays. It has been reported that the interaction between the aster and cortex is mediated by the dynactin complex and cytoplasmic dynein (Busson et al., 1998; Skop and White, 1998). Therefore, it is possible that a similar mechanism was involved in the pointing process of the sperm diaster in the starfish eggs. It is considered that only one pole of MA interacts with a site at some cortex to make its axis position and point during asymmetric division (Strome, 1993). However, it is quite likely that both asters of the sperm diaster interact with the animal cortex for orientation and the axis of the sperm diaster becomes perpendicular to the animal–vegetal axis, which results in equal division as early cleavages in starfish eggs undergo equal division. It has been reported in mouse eggs that the orientation of the first cleavage plane was determined by the midbody which formed during meiosis (Plusa et al., 2002).

However, the presumptive cleavage plane was not through the site where polar bodies and midbodies formed in the meiotic spindle-shifted eggs of the starfish (Stephano and Gould, 2000). We propose another model that the gradient along the animal–vegetal axis in the cytoplasm is involved in the pointing process of the sperm diaster (Fig. 10b). Both asters of the sperm diaster were attracted to the animal pole according to the axial gradient. The gradient like this was proposed by Hörstadius (1973), a vegetalizing gradient with its maximal activity at the vegetal pole, and an animalizing gradient with its maximal activity at the animal polar region. In addition, the sperm asters increased in size with the distance from the animal pole in polyspermic eggs (Stephano and Gould, 2000). They suggested that this phenomenon was caused by a gradient of MAPK activity along the animal–vegetal axis.

Further research is needed to clarify the orientation mechanism for the first MA: transplanting the animal pole cortex to such eggs as GV-eliminated eggs would clarify the animal cortical factor model. In case the sperm diaster interacts with the animal cortex to orient to the animal pole, the sperm diaster should point to the transplanted animal cortex. Moreover, depolymerization astral rays would clarify the animal cortical model easily. In case the spindle is anchored to the cortex by astral microtubules, the addition of low doses of microtubule depolymerizing reagents such as nocodazole should randomize spindle azimuth. These
experiments would provide functional insight to the mechanism of spindle orientation.

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References


