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Centrosome destined to decay in starfish oocytes

Yukako Shirato[1,2,*], Miwa Tamura[1,2], Mitsuki Yoneda[3] and Shin-ichi Nemoto[1,2,†]

In contrast to the somatic cell cycle, duplication of the centrioles does not occur in the second meiotic cycle. Previous studies have revealed that in starfish each of the two centrosomes in fully-grown immature oocytes consists of two centrioles with different destinies: one survives and retains its reproductive capacity, and the other is lost after completion of meiosis. In this study, we investigated whether this heterogeneity of the meiotic centrioles is already determined before the re-initiation of meiosis. We prepared a small fragment of immature oocyte containing the four centrioles and fused it electrically with a mature egg in order to transfer two sets of the premeiotic centrioles into the mature cytoplasm. Two asters were present in this conjugate, and in each of them only a single centriole was detected by electron microscopy. In the first mitosis of the conjugate artificially activated without sperm, two division poles formed, each of which doubled in each subsequent round of mitosis. These results indicate that only two of the four premeiotic centrioles survived in the mature cytoplasm and that they retained their reproductive capacity, which suggests that the heterogeneity of the maternal centrioles is determined well before re-initiation of meiosis, and that some factor in the mature cytoplasm is responsible for suppressing the reproductive capacity of the centrioles destined to decay.

KEY WORDS: Centrioles, Centrosomes, Centrosome regulation, Centrosome duplication, Electrofusion, Maturation division, Meiosis, Starfish

INTRODUCTION

Artificial parthenogenesis in starfish was pioneered in the early 1980s by Obata and Nemoto (Obata and Nemoto, 1984) and later, Washitani-Nemoto et al. (Washitani-Nemoto et al., 1994) found that suppression of polar body (PB) extrusions in artificially activated oocytes induces parthenogenetic development, whereas eggs that matured normally did not develop, even with artificial activation. They suggested that the meiotic centrosomes retained in the eggs by the failure of PB extrusion are diverted to mitosis-organizing centers in the mitotic spindle, resulting in parthenogenetic development.

Washitani-Nemoto et al. (Washitani-Nemoto et al., 1994), Uetake et al. (Uetake et al., 2002) and Zhang et al. (Zhang et al., 2004) utilized the suppression of PB extrusion as a useful tool for analysing the mechanism of the 'paternal inheritance of the centrosomes in development', first noted by Boveri (Boveri, 1887). As we know, control of the centrosome inheritance is an issue of fundamental importance for all sexually reproductive organisms.

Centrosomal behaviour during normal meiosis in starfish oocytes is shown in Fig. 1. According to Sluder et al. (Sluder et al., 1989) and Kato et al. (Kato et al., 1990), each pole of a meiosis-I spindle in starfish oocytes has a pair of centrioles (Fig. 1B), but only one centriole is found in each pole of the meiosis-II spindle (Fig. 1D). In other words, the centrioles are not duplicated during meiosis II. Of the four centrioles in meiosis I, two of them are inherited by the first PB (PB1), another one by the second PB (PB2), and the remaining one by the mature egg during meiosis (Fig. 1E).

[1]Department of Biology, Faculty of Science, Ochanomizu University, Bunkyo, Tokyo 112-8610, Japan. [2]Tateyama Marine Laboratory, Marine and Coastal Research Center, Ochanomizu University, Koh-yatsu, Umi-no-Hoshi, Tateyama, Chiba 294-0301, Japan. [3]Takiyama 5-7-7, Higashikurume, Tokyo 203-0033, Japan.

*Present address: Division of Biology, Department of Life Sciences, Graduate School of Arts and Sciences, University of Tokyo, Komaba, Meguro-ku, Tokyo 153-8902, Japan
†Author for correspondence (e-mail: snemo@cc.ocha.ac.jp)

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Uetake et al. (Uetake et al., 2002) used starfish oocytes that had formation of their PB suppressed to investigate the behaviour of all the maternal centrosomes/centrioles throughout meiosis. When the two pairs of meiosis-I centrioles were retained in the oocyte by suppression of both PB1 and PB2 extrusion ('0pb egg'), they separated into four single centrioles in meiosis II, but after completion of the meiotic process, only two were found with the pronucleus in the mature egg. When the two centrioles of a meiosis-II spindle were retained in the oocyte by suppression of PB2 extrusion alone ('1pb egg'), only one was found after meiosis. When these PB-suppressed eggs (0pb and 1pb eggs) were artificially activated, all the surviving centrioles duplicated to form pairs, eventually organizing into mitotic spindles. Those findings demonstrated that there is heterogeneity in the survival and reproductive capacity of the maternal centrioles and that the centrosomes with the reproductive centrioles are selectively cast off into the PB (PB1 and PB2), resulting in a mature egg inheriting a non-reproductive centriole that would degrade after the completion of meiosis (Fig. 1E). Uetake et al. (Uetake et al., 2002) thus introduced the concept of 'nonequivalence' of maternal centrioles.

Tamura and Nemoto (Tamura and Nemoto, 2001) had earlier examined the reproductive capacity of the centrosomes in PB1 or PB2 by transplanting them into artificially activated eggs, which revealed that one of the two centrioles in PB1 and the sole centriole in PB2 are reproductive and able to form bipolar spindles leading to cleavage and subsequent parthenogenetic development. Based on their results, they also suggested that the four maternal centrioles are heterogeneous in their reproductive capacity.

Such 'nonequivalence' or 'heterogeneity' among the maternal centrioles, however, does not become apparent until the completion of meiosis and an exploration of the mechanisms regulating the centrioles in meiosis, has to address two questions: (1) At what stage of meiosis are the fates of the centrioles determined? (2) What conditions are needed for the loss of function ('degradation') of half of the centrioles?

Our hypothesis was that the fate of the centrioles is determined before the resumption of meiosis, and that some factor in the cytoplasm of mature eggs is responsible for inducing the degradation

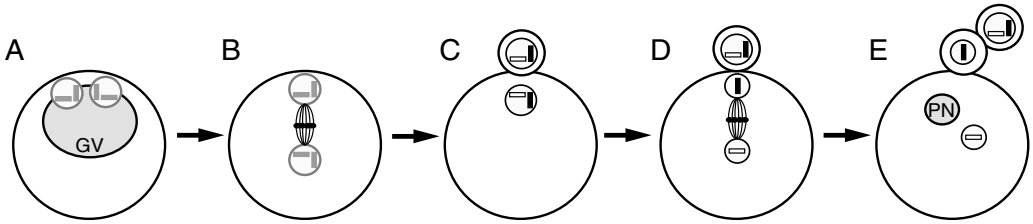

Fig. 1. Centrosomal behaviour during normal meiosis of a starfish oocyte, based on the experimental results by Tamura and Nemoto (Tamura and Nemoto, 2001) and Uetake et al. (Uetake et al. 2002). (**A**) Fully-grown immature oocyte with the germinal vesicle (GV). (**B**) Metaphase I. (**C**) The first polar body (PB1) extruded. (**D**) Metaphase II. (**E**) The second polar body (PB2) extruded. The pronucleus (PN) formed. Solid rectangles are reproductive centrioles. Open rectangles are centrioles destined to decay after completion of meiosis. Gray rectangles in A and B are centrioles to be characterized in the present study.

of the centrioles. In order to test our theory, we developed a new technique for investigating the reproductive capacity of the centrioles. (In this paper we use the term 'to degrade', to mean that centrioles lose their capacity to function as the mitotic division poles.)

MATERIALS AND METHODS

Experimental protocol

Immature oocytes, arrested in early prophase of meiosis I, contain two asters (premeiotic asters) located between the eccentric germinal vesicle (GV) and the animal pole (Otto and Schroeder, 1984; Picard et al., 1988; Miyazaki et al., 2000). The basis of our new experimental procedure is that the centrosomes are in the loci of the respective premeiotic asters (Uetake et al., 2002).

First, we bisected an immature oocyte (Fig. 2A,B) and removed the GV in the nucleated half with a micropipette (Fig. 2C). The resultant enucleated fragment should retain the pair of premeiotic asters, each with a centrosome at the center. The fragment was then subjected to electric fusion with a mature egg (Fig. 2E), so that the premeiotic centrosomes were suddenly transferred into the mature cytoplasm, without experiencing meiotic divisions. These 'heteroplasmic conjugates' (Fig. 2F) were the material for the present study. In one experiment, they were artificially activated without sperm, and then continuously observed by light microscopy for the emergence of single asters or mitotic figures and the occurrence of nuclear divisions. In another experiment, non-activated conjugates were examined by transmission electron microscopy for the number of surviving centrioles.

Oocyte preparation

Oocytes of the starfish *Asterina pectinifera* during the breeding season in spring–summer were used. To obtain follicle-free immature oocytes arrested at prophase of meiosis I, isolated ovaries were treated with Ca^{2+}-free artificial seawater and then transferred into filtered natural seawater to induce spawning of oocytes (Nemoto et al., 1980).

Preparation of the 'centrosome-bearing fragments'

The oocytes in seawater were placed in a dish coated with 1% agar and each one was manually bisected with a fine glass needle into an animal (GV-containing) and vegetal (non-nucleated) fragment (Kiyomoto and Shirai, 1993). The GV-containing fragment was kept as small as possible (Fig. 2B). A micropipette connected to a microinjector (IM-5B; Narishige, Tokyo, Japan) on a micromanipulator (NO-202, Narishige) was then inserted into the GV-containing fragment, opposite the animal pole (Fig. 2C), and the GV was very slowly and continuously aspirated out into the micropipette according to the procedure of Miyazaki et al. (Miyazaki et al., 2000). The size of the fragments was further reduced to about 100 μm in diameter by enucleation, resulting in a volume that was about 25% that of an intact oocyte (160 μm in diameter). An essential feature of our technique is the transfer of the two premeiotic centrosomes into the cytoplasm of a mature egg, with minimal transfer of immature cytoplasm, which is the reason for reducing the size of the non-nucleated fragment. To remove both the jelly layer and the vitelline coat, the fragments were treated with 0.01% actinase (Kaken Pharmaceutical, Tokyo, Japan) in seawater for 10-15 minutes and rinsed several times in seawater before their use as centrosome donors.

Determining the presence of meiotic centrosomes in the fragments

Miyazaki et al. (Miyazaki et al., 2000) showed that oocytes retain a pair of premeiotic asters even after aspiration of the GV. To confirm that the two centrosomes in the loci of the premeiotic asters were retained in our fragments, we carried out indirect immunofluorescence staining using an anti-γ-tubulin antibody, the specific probe for centrosomes, according to the methods of Uetake et al. (Uetake et al., 2002). The fragments deprived of the vitelline coat after treatment with 0.01% actinase were immersed in an extraction medium, plated onto glass slides, fixed with 6% paraformaldehyde and incubated overnight with the rabbit anti-γ-tubulin polyclonal antibody (T3559, Sigma-Aldrich Co., St Louis, MO, USA). Next, the samples were stained with a Texas Red-labelled goat

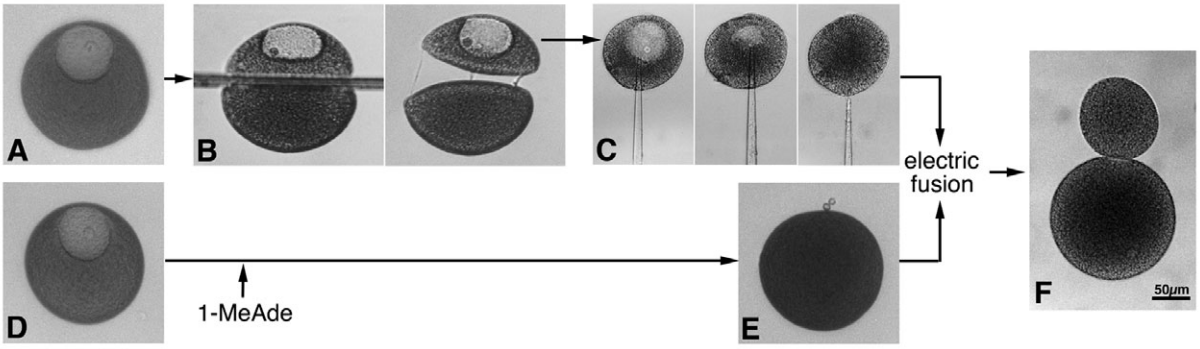

Fig. 2. Experimental protocol. (**A**) Immature oocyte. (**B**) The immature oocyte is bisected manually with a fine glass needle into a GV-containing and a non-nucleate fragment. (**C**) The GV-containing fragment is enucleated with a micropipette. This fragment is used as a centrosome donor. (**D**) Actinase-treated immature oocyte that is treated with 1-methyladenine (1-MeAde) to induce maturation. (**E**) Mature egg bearing both PB1 and PB2. (**F**) Conjugate of a non-nucleate fragment and a mature egg. Scale bar: 50 μm.

anti-rabbit IgG antibody (Biosource International, Camarillo, CA, USA) and examined with a fluorescence microscope (OPTIPHOT, Nikon, Tokyo, Japan). The two centrosomes appeared as two spots in the fragment (Fig. 3).

Preparation of mature eggs as centrosome recipients

Immature oocytes were first subjected to actinase treatment (0.01%), and then treated with 3 μM 1-methyladenine (1-MeAde; Sigma-Aldrich Co.) to induce maturation (Kanatani, 1969). Mature eggs with both PB1 and PB2 (cf. Fig. 2E) were used for the electric fusion process. It is known that a normally matured egg with both PB1 and PB2 will not cleave even after activation without sperm, indicating the absence of reproductive centrioles (Obata and Nemoto, 1984; Washitani-Nemoto et al., 1994; Tamura and Nemoto, 2001; Uetake et al., 2002).

Electric fusion of fragments and mature eggs

A chamber for electric fusion designed by Yoneda (Yoneda, 2000) was filled with a 0.88 M solution of mannitol with 0.4 mM $CaCl_2$ and 0.1 mM $MgSO_4$ (Yoneda, 1997). One fragment and one mature egg were transferred into the chamber and placed side by side in the center along the line of the electric field between the two planar electrodes. Each round of electric pulses was routinely four repetitions of a pulse sequence comprising a high frequency AC field and a brief rectangular DC pulse (Yoneda, 1997). The frequency of the AC field was fixed at 2.5 MHz. The peak-to-peak amplitude was 200 Vp-p/cm. The duration of each sequence was 10-15 seconds. The duration of the brief rectangular DC pulse was fixed at 50 μseconds. The voltage of the DC pulse was 250-290 V/cm.

To date, two fusion procedures have been reported, one for fusing two immature oocytes and another for fusing two maturing oocytes (Yoneda, 1997; Yoneda, 2000; Masui et al., 2001). In the present study, we developed a new technique for fusing an immature oocyte and a mature egg, as explained in the Results.

Artificial activation of conjugates

The fusion product, or 'conjugate', was activated with 10 μM calcium ionophore A 23187 (Calbiochem-Novabiochem, La Jolla, CA, USA) for 10 minutes, rinsed several times in seawater and then allowed to develop.

Light microscopy

A microscope equipped with both differential interference-contrast and polarization optics (HPD; Nikon, Tokyo, Japan) was used. Microphotographs were taken with Neopan 400 Presto film (Fuji Photo Film, Tokyo, Japan).

Transmission electron microscopy

Following the procedure of Kato et al. (Kato et al., 1990), each conjugate was washed briefly with 0.53 M NaCl solution and fixed with glutaraldehyde-OsO_4 mixture [1% glutaraldehyde, 1% OsO_4 and 0.45 M sodium acetate in 0.05 M sodium phosphate buffer (pH 6.4)] for 20 minutes at room temperature. After dehydration in an ethanol series, the conjugates were stained en bloc with uranyl nitrate and lead acetate, and then embedded in Poly/Bed 812 (Polyscience Inc., Warrington, UK) on a flat plate of silicone rubber. The blocks were trimmed to an area of approximately 5 mm and serially sectioned at 0.15 μm thickness with an ultramicrotome (Ultracut UCT, Leica, Wien, Austria). However, because the present conjugates were very large (up to 200 μm in diameter) and there was not a natural marker of the loci of the asters, we began making serial 1 μm-thick sections until we found a very faint radial structure, or trace of the aster, and then began thin sectioning. The thin sections were examined in an electron microscope (JEM-1230, JEOL, Tokyo, Japan) to determine the number of centrioles in each of the asters.

RESULTS

Fusion and post-fusion process

Under our experimental conditions, maturing oocytes without a jelly layer or vitelline coat extruded PB2 about 90 minutes after 1-MeAde treatment and about 40 minutes later the mature egg was subjected to electric fusion with a fragment. We began with a few rounds of fusion pulses with fixed polarity of the DC pulse so that the mature egg faced the positive electrode (anode) and consequently the fragment was close to the negative electrode (cathode). The rounds of fusion pulses were repeated until a very small bulge formed on the surface of the fragment that was in contact with the mature egg. We then changed the circuit so that the polarity of the DC pulse was reversed alternately in each sequence (Yoneda, 1997) until a bulge formed on the surface of the mature egg where it was in contact with the bulge on the fragment surface. The bulges then fused, which lead to fusion of the entire fragment and mature egg. It took 3-10 minutes to create the heteroplasmic conjugate and up to three conjugates were often obtained at a time. Immediately after the fusion, the conjugates were removed from the fusion chamber for a brief rinse with fresh seawater, and then activated by a 10-minute treatment with calcium ionophore. The narrow neck joining the pair gradually broadened and they eventually formed a single sphere.

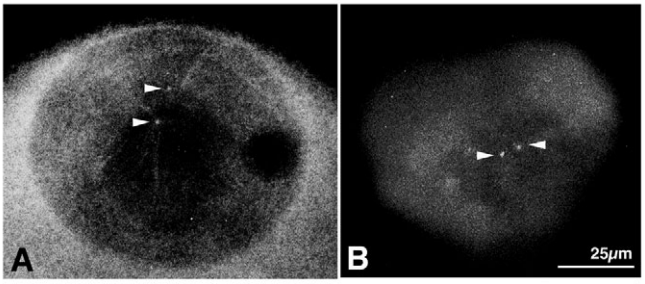

Fig. 3. Immunofluorescent staining with an anti-γ-tubulin antibody of a non-nucleate fragment. (**A**) Whole immature oocyte. Two spots (arrowheads) stained by the anti-γ-tubulin antibody are located between the GV and the animal pole. (**B**) Enucleated fragment. Two spots (arrowheads) are stained by the anti-γ-tubulin antibody. Scale bar: 25 μm.

It is known that the chain of electric pulses for fusion may activate some mature eggs, as evidenced by the breakdown of the pronuclear envelope, which takes place about 1 hour later (Yoneda, 1997; Yoneda, 2000). In the case of our conjugates, incidental activation by the fusion pulse alone may cause the cleavage of the first mitotic cycle. In our experimental protocol the purpose of starting the ionophore treatment immediately (within 10 minutes) after the fusion was to cancel any effect of precocious activation by the fusion pulses.

Development of activated conjugates

On activation with calcium ionophore, the conjugates underwent a cycle of cleavages (Fig. 4), the first cleavage furrow appearing about 60 minutes after activation. Often the furrow regressed (Fig. 4A) and the egg remained as a single cell (Fig. 4B). At the time of the next cycle such eggs directly divided into four blastomeres (Fig. 4C,D) and the third cleavage formed eight blastomeres (Fig. 4E). The cleavage interval was about 40 minutes, which is similar to normal embryos.

We consider that it was the fusion with the centrosome-bearing fragment that enabled the activated egg to begin the cycle of cleavages, indicating that the premeiotic centrosomes in the fragment were diverted into the mitosis-organizing centers of the conjugates.

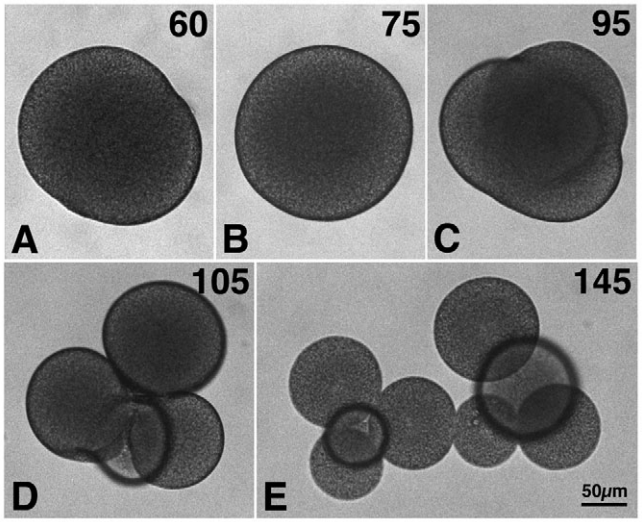

Fig. 4. Development of a conjugate at 22°C. The time (minutes) after activation is given in the upper right corner of each image. (**A**) The first cleavage. (**B**) The furrow regresses, and the conjugate remains as a single cell. (**C**) The second cleavage. Multiple furrows appear. (**D**) Four blastomeres form at the second cleavage. (**E**) The third cleavage forms eight blastomeres. Scale bar: 50 μm.

Nuclear events and formation of mitotic asters in the conjugates

For detailed observation of nuclear events and the formation of mitotic asters, activated conjugates in 80% seawater were compressed to 60 μm thickness between a glass slide and cover slip. The compression enabled precise timing of the nuclear changes, although it inhibited the formation of the cleavage furrow. When microscopic observation started about 5 minutes after activation at 20°C, the female pronucleus was usually retained. If it was not, we discontinued observing these conjugates, because they must have been activated spontaneously long before the ionophore activation.

The breakdown of the pronuclear envelope (NEBD) in 28 conjugates took place 56±8 (s.d.) minutes after activation, so the conjugates underwent the first mitosis about 1 hour after activation (compare Fig. 5B, Fig. 6B, Fig. 7B), which is similar to the mitotic time schedule of intact eggs fertilized after completion of meiosis at 20°C (see Nomura et al., 1993).

Within 7±3 (mean ± s.d.) minutes of NEBD, two asters suddenly became visible and their loci varied among the conjugates: in some, both asters formed near where the pronucleus had been located, in others, only one aster formed near the site of the pronucleus and the other aster formed at a distance from the nuclear site, and in still other conjugates both asters were located apart from the nuclear site. We designated these three patterns of the location of the formed asters as Patterns 1, 2, and 3 (Figs 5-7). A common feature of all three patterns so far observed was that the number of asters forming at the first mitosis was always two (Fig. 8).

Pattern 1 (8 conjugates)

As shown in Fig. 5, polarization microscopy revealed that each of the asters formed a spindle aster, and a bipolar spindle formed at first mitosis. Two nuclei then emerged. After the breakdown of the two nuclei in the next round of mitosis, two bipolar spindles were assembled and four nuclei formed. In the third round, the four nuclei broke down and four bipolar spindles appeared, resulting in formation of eight nuclei. Thus in each of the cycles, the number of division poles and nuclei doubled (Fig. 8).

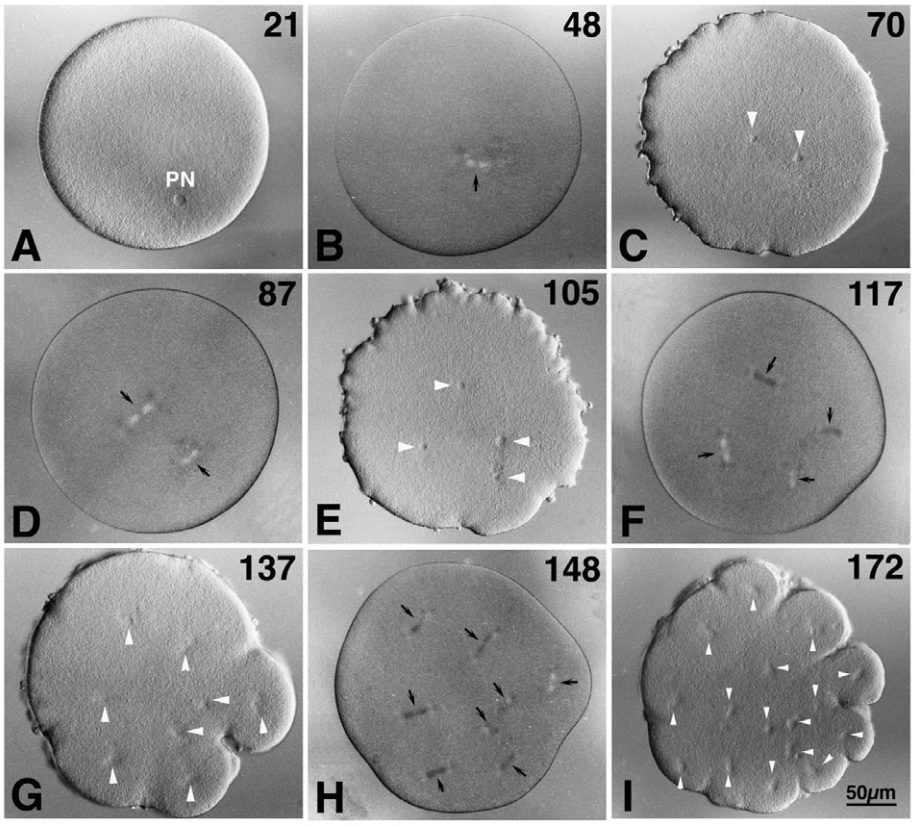

Fig. 5. Nuclear events and the formation of mitotic asters in a Pattern 1 conjugate. The time (in minutes) after activation is given in the upper right corner of each image. Black arrows indicate bipolar spindles. White arrowheads indicate nuclei. (**A**) Pronucleus (PN) formation. (**B**) The first mitotic cycle. A bipolar spindle develops. (**C**) Two nuclei form. (**D**) The second mitotic cycle. Two bipolar spindles develop. (**E**) Four nuclei form. (**F**) The third mitotic cycle, forming four bipolar spindles. (**G**) Eight nuclei form. (**H**) The fourth mitotic cycle, forming eight bipolar spindles. (**I**) Sixteen nuclei form. (A,C,E,G,I) Differential interference-contrast microscopy; (B,D,F,H) polarization microscopy.

Pattern 2 (4 conjugates)

At first mitosis, a monopolar (half) spindle formed at the nuclear site (Fig. 6) with the other aster remaining at a distance. A nucleus then formed at the site of the monopolar spindle and following its breakdown in the next round of mitosis, a bipolar spindle formed and two nuclei then formed. The isolated aster had now doubled. In the third round, the two nuclei broke down and two bipolar spindles formed, resulting in formation of four nuclei. The number of isolated asters was now four. Thus in each of the cycles, the number of asters doubled (Fig. 8).

Pattern 3 (12 conjugates)

As shown in Fig. 7, the two asters were located apart and at a distance from the nuclear site. At the site where the pronucleus had been located, an aster-like structure appeared in first mitosis and subsequently a nucleus formed there. Following the breakdown of the nucleus in the next round of mitosis, each of the two separated asters doubled to form four asters. An aster-like structure again appeared at the nuclear site and one nucleus reformed in the same position as the aster-like structure. In the third round of nuclear breakdown, the four isolated asters doubled to form eight asters, but the reformed aster-like structure remained single. In the fourth round of nuclear breakdown, the eight isolated asters doubled to form 16 asters. Thus the number of asters doubled in each of the cycles (Fig. 8).

We have thus classified 24 conjugates into Patterns 1, 2 and 3. The remaining 4 of the 28 conjugates failed to undergo the second round of mitosis and were excluded from the analysis.

A feature specific to Pattern 3 conjugates is the appearance of 'aster-like structure'. We are confident that this structure is unrelated to the premeiotic centrosomes. A brief notes on the aster-like structure is given later, in the Discussion.

Number of asters and centrioles in the conjugates before ionophore activation

For further analysis of the heterogeneity among meiotic centrioles, we needed to know the number of surviving centrioles in our conjugates. We kept formed conjugates in seawater without calcium-ionophore activation. If the conjugates are incidentally activated by fusion pulses alone, they would undergo the first cleavage about 1 hour later (cf. Fig. 4A). Therefore, to avoid using those conjugates that had been incidentally activated by fusion pulses, we routinely waited more than 120 minutes and selected those conjugates that were undivided and retained their spherical profile. They were then subjected to fixation for transmission electron microscopy.

It was very difficult to detect the faint trace of a single aster on the thick sections, but with practice, we succeeded in locating two asters in one conjugate. They were about 40 μm apart (Fig. 9) and in the center of each aster we found a single centriole, which indicates that, of the four centrioles derived from the immature oocyte, two survived in the mature cytoplasm of the conjugates, and the remaining two 'degraded', i.e. they lost the ability to organize the mitotic asters.

DISCUSSION

Heterogeneity of the centrioles in immature oocytes

Based on our results, we are now certain that the premeiotic centrosomes were recruited to the mitosis-organizing centers when suddenly introduced into the mature cytoplasm of the fused conjugate. The timing of the mitotic cell cycle was similar to that in normally fertilized eggs. The number of the asters found at the first mitosis was always two, but the pattern of the location of asters with respect to the nucleus was diverse among conjugates (Figs 5-8).

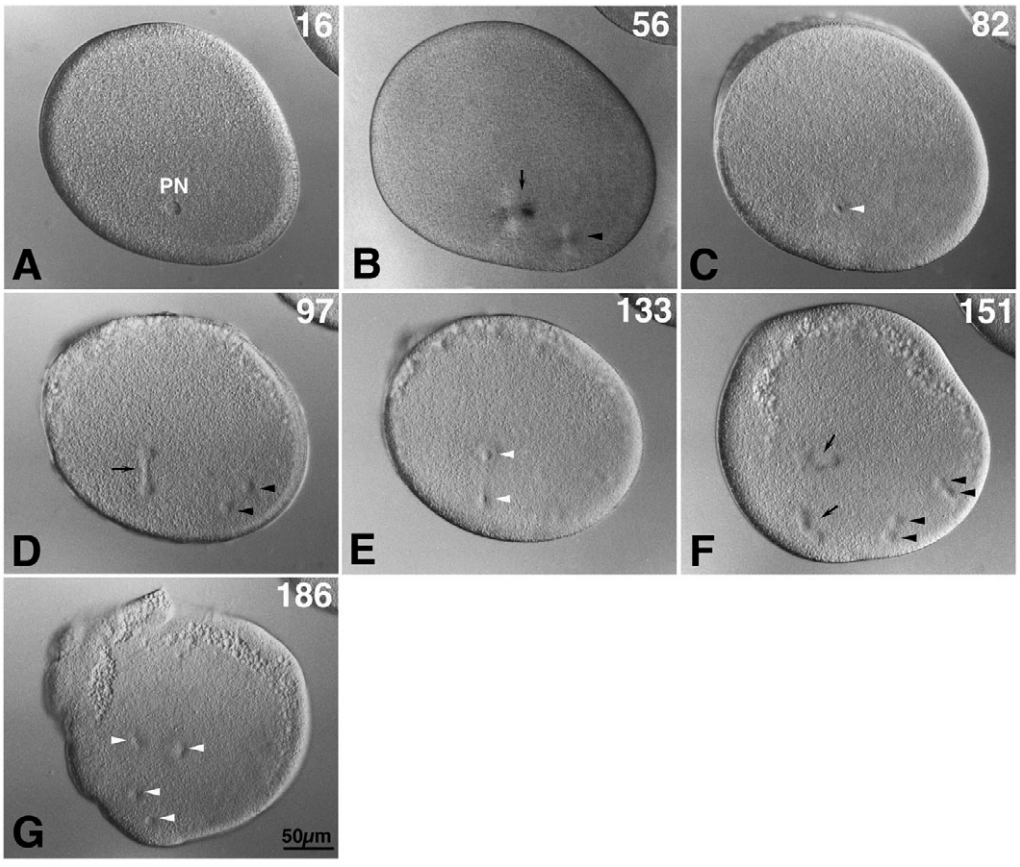

Fig. 6. Nuclear events and the formation of mitotic asters in a Pattern 2 conjugate. The time (in minutes) after activation is given in the upper right corner of each image. Black arrows indicate monopolar or bipolar spindles; white arrowheads indicate nuclei; black arrowheads indicate single asters. (**A**) Pronucleus (PN) formation. (**B**) The first mitosis. One monopolar spindle and one aster develop [see Tamura and Nemoto (Tamura and Nemoto, 2001) for clearer pictures of monopolar spindles]. (**C**) One nucleus forms. (**D**) The second mitotic cycle. One bipolar spindle and two asters develop. (**E**) Two nuclei form. (**F**) The third mitotic cycle, forming two bipolar spindles and four asters. (**G**) Four nuclei formed. (a,c,e,g) Differential interference-contrast microscopy; (b,d,f) polarization microscopy.

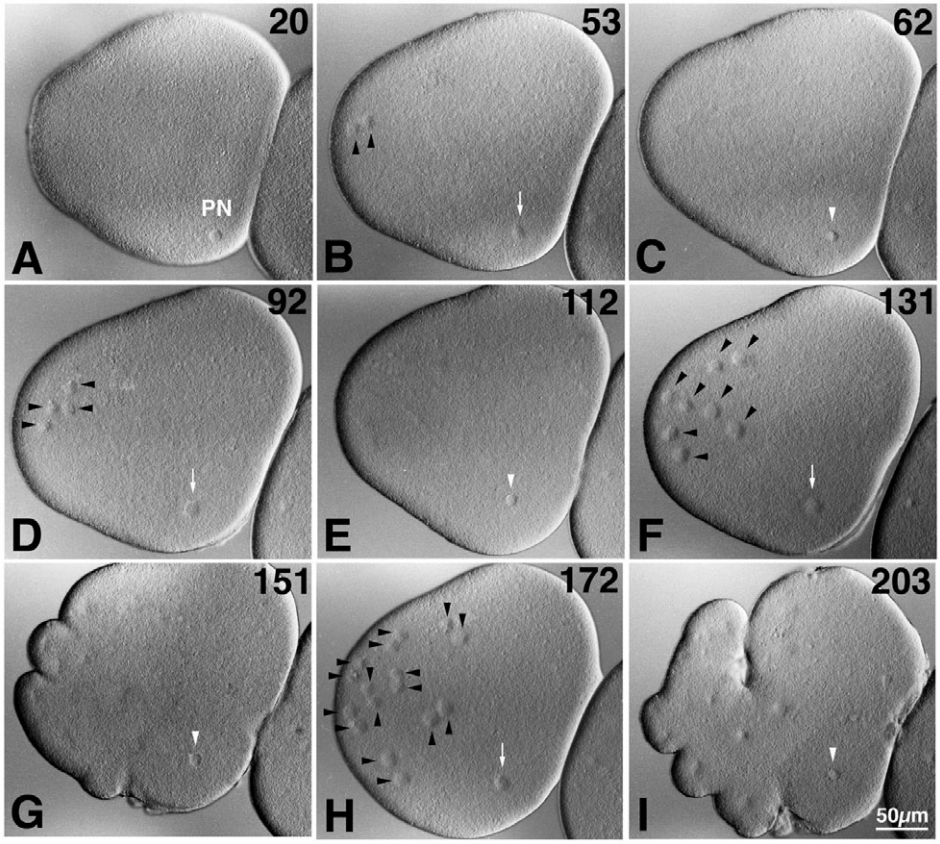

Fig. 7. Nuclear events and the formation of mitotic asters in a Pattern 3 conjugate. The time (in minutes) after activation is given in the upper right corner of each image. White arrows indicate 'aster-like structures'; white arrowheads indicate nuclei; black arrowheads indicate single asters. (**A**) A pronucleus (PN) forms. (**B**) The first mitotic cycle. An 'aster-like structure' developed at a site where the PN had been located. Two asters emerge, away from the pronuclear site. (**C**) One nucleus reforms. (**D**) The second mitotic cycle. Four asters and a single 'aster-like structure' develop. (**E**) One nucleus reforms. (**F**) The third mitotic cycle forms eight asters and an 'aster-like structure'. (**G**) One nucleus reforms. (**H**) The fourth mitotic cycle, forms 16 asters and an 'aster-like structure'. (**I**) One nucleus reforms. (a,c,e,g,i) Differential interference-contrast microscopy; (b,d,f,h) polarization microscopy.

For simplicity of discussion, we will take Pattern 1 as a 'typical' case in which the two asters emerged near the site where the egg pronucleus had been located, each aster forming one pole of the bipolar spindle in the first nuclear division. As shown in Fig. 5, the number of bipolar spindles increased in a 2-4-8 fashion in each of the subsequent cycles.

In the so-called '0pb eggs', in which formation of both PB1 and PB2 is suppressed, Uetake et al. (Uetake et al., 2002) confirmed that the maternal centrosomes/centrioles form a bipolar mitotic spindle at the same stage during the first mitosis as in normally fertilized eggs. In this respect the behaviour of the mitotic asters in our Pattern 1 conjugates replicated that of the asters in 0pb eggs. Uetake et al. (Uetake et al., 2002) found that two centrioles survive after the completion of meiosis and that each of the two surviving centrioles in 0pb eggs reproduces during the first S phase, and in fact they noted a pair of centrioles with an orthogonal configuration in each of the two centrosomes forming the bipolar spindle at the first mitosis. It is thus reasonable to suppose that the poles of the bipolar spindle at the first mitosis in the present conjugates also had paired centrioles. The regular doubling of the bipolar spindles in the second and third cell cycles (Fig. 5) infers the presence of paired centrioles

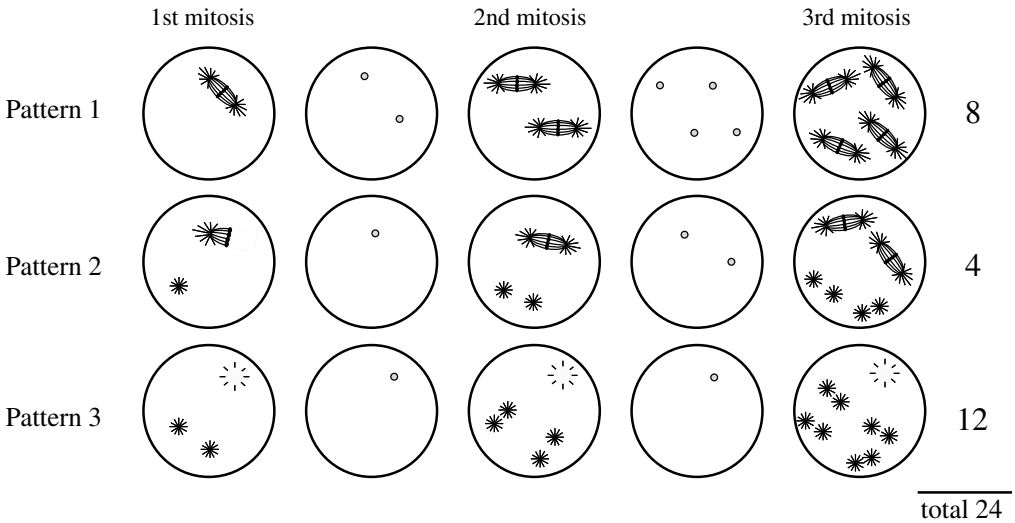

Fig. 8. Schemata of nuclear events and the formation of mitotic asters after activation in the conjugates.

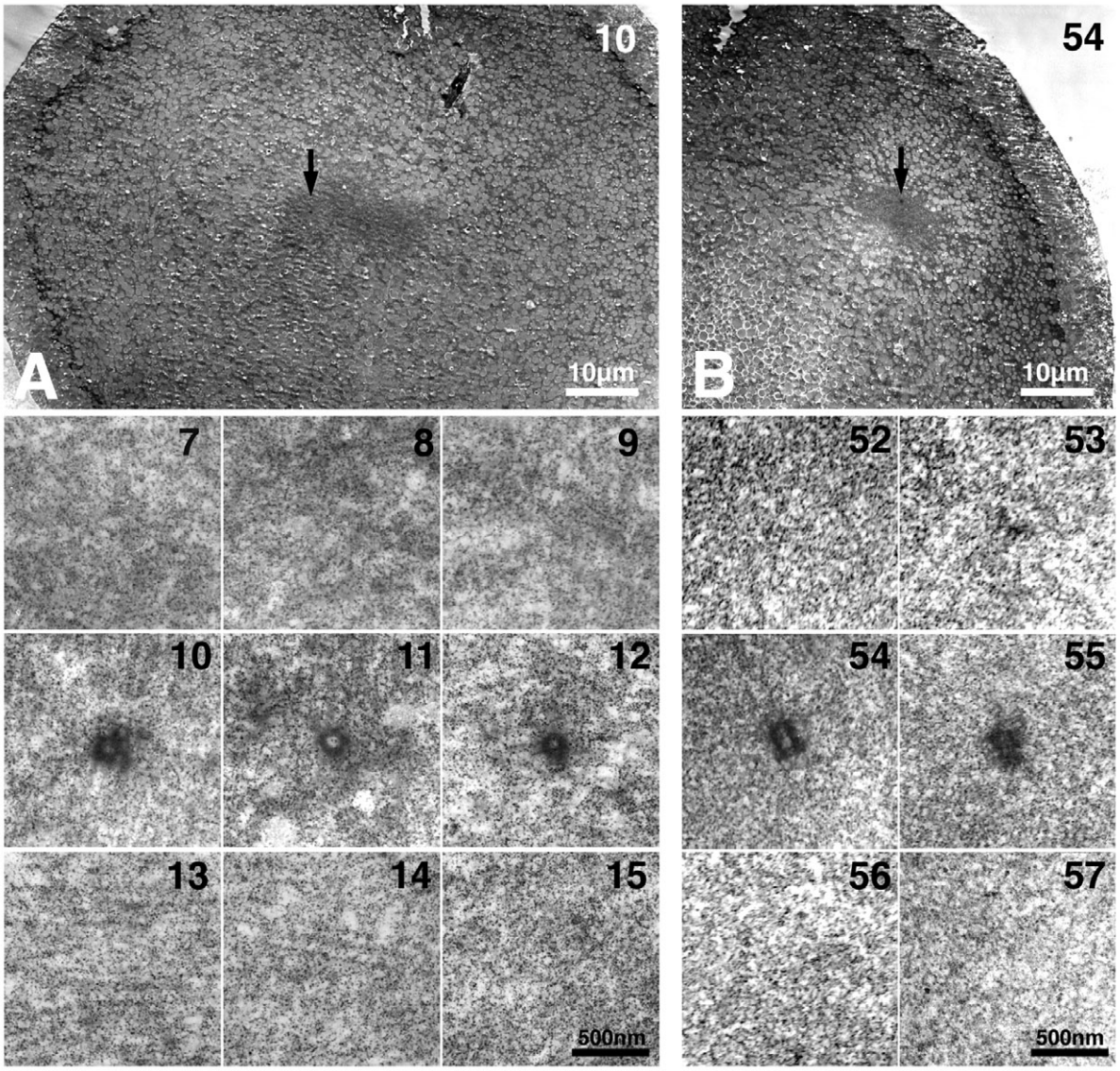

Fig. 9. Electron micrographs of a non-activated conjugate with two single asters. Each of the asters (**A**,**B**) contains one centriole at the center. The arrows point to the center of the respective aster. Numerals in the upper right corner of each frame indicate the number of the serial thin section (each 0.15 μm thick).

in each pole of the spindle at first mitosis and therefore the number of centrioles in our conjugates at first mitosis would be four. We have demonstrated one centriole in each of the two asters in a non-activated conjugate at the pronuclear stage (Fig. 9), so there is no doubt that the two centrioles replicated once, probably during the first S phase initiated by activation of the conjugates.

We now examine the cases of Pattern 2 and Pattern 3 conjugates. In these conjugates the bipolar spindle did not form at the first mitosis and we believe that its failure to form is simply because one or two of the asters emerged at a distance from the female pronucleus. Fusion of a non-nucleated oocyte fragment bearing premeiotic centrosomes with a mature egg containing a pronucleus will result in the centrosomes and the pronucleus initially being located apart. Activation of the conjugates will induce them to move together, as is the case in a normally fertilized egg in which the sperm aster and the female pronucleus move toward each other for syngamy. However, we compressed the conjugates for observation by light microscopy and this would hinder the rapid movement required for the aster and the pronucleus to come together by the time of the first mitosis. This appears to have caused the occurrence of Pattern 2 and Pattern 3 conjugates.

However, the number of asters that formed at the first mitosis was invariably two, common to all three patterns. Moreover, we observed, in all three patterns, that each of the asters doubled at each of the subsequent mitotic cycles. We consider that the results obtained in the Pattern 2 and Pattern 3 conjugates also support our conclusion that only two of four centrioles survive.

What is remarkable about the maternal centrioles in our conjugates is that they had not undergone meiotic divisions and had not contributed to the formation of the meiotic spindles and yet, of the four centrioles only two survived with the capacity to replicate. Hence we conclude that their fate was determined while they were in the fully-grown immature oocyte, well before the resumption of meiosis.

Possible structural heterogeneity of the centrioles

Recent studies by Tamura and Nemoto (Tamura and Nemoto, 2001) and Uetake et al. (Uetake et al., 2002) on artificial parthenogenesis in starfish introduced the concept of 'heterogeneity' or 'nonequivalence' of the reproductive capacity of the maternal centrioles. A typical example is the meiosis-II spindle: one pole of the spindle positioned beneath the cell surface is inherited by the forming PB2, and the other pole, located in the deeper cytoplasm, is left in the mature egg. Each pole contains a single centriole. The studies showed that the PB2 centriole has reproductive capacity, whereas the egg centriole is lost after the completion of meiosis. How the pole of the reproductive centriole selectively locates itself beneath the cell surface to be cast off into the PB2 is a newly raised question. Tamura and Nemoto (Tamura and Nemoto, 2001) and Uetake et al. (Uetake et al., 2002) consider that it has a device for anchoring itself to the cell surface, a structure unique to the reproductive centriole. Such 'structural heterogeneity' must be linked to the heterogeneity in reproductive capacity.

Thus, in order for the pole of the meiosis-II spindle containing the reproductive centriole to be correctly positioned beneath the cell surface, the fate of the centriole has to have been determined by the time of meiosis II. This was confirmed in the present study. Actually we found that the fate of the centriole was already fixed at the stage of the fully-grown immature oocyte. Whether the time its fate is determined can be traced back further to an even earlier stage of oogenesis is a subject for future study.

Process of degradation of the maternal centrioles

In the case of 0pb/1pb eggs, the 'nonreproductive centrioles' are lost shortly after the completion of meiosis (Uetake et al., 2002). Nuclear events, such as the formation of the pronucleus or cell-cycle arrest at the G1 phase, arising just after the completion of meiosis, suggest changes in the egg cytoplasm that trigger these events. Uetake et al. (Uetake et al., 2002) argue that the supposed changes in the cytoplasm 'may be related to the suppression of some maternal centrosomes/centrioles'. A similar suppression was also observed in our conjugates. The maternal centrioles transferred directly into the mature cytoplasm had not undergone meiotic divisions, yet two centrioles degraded. We anticipate that the cytoplasmic environment of the mature egg is the necessary condition for inducing the destined centrioles to decay.

Notes on the 'aster-like structure'

Tamura and Nemoto (Tamura and Nemoto, 2001) described a similar structure ('monaster') that formed at the site of the pronucleus in intact eggs artificially activated without sperm. It emerged at each mitotic cycle, never duplicated and remained single. Sluder et al. (Sluder et al., 1989) also observed the formation of such a monaster in fertilized starfish eggs when syngamy of the sperm and egg pronuclei was artificially prevented. Based on the common morphology of our 'aster-like structure' and the monaster, their site of appearance, and the timing of their formation, we regard them as identical. Uetake et al. (Uetake et al., 2002) demonstrated that the monaster in activated intact eggs does not have a region recognized by anti-γ-tubulin antibody, indicating the absence of a centrosome. We have also recently observed (unpublished data) that injection of the antibody into fertilized eggs inhibits aster formation by the sperm centrosome, but does not inhibit the formation of the monaster. Zhang et al. (Zhang et al., 2004) argue that, in the monaster, chromosomes locate to its center (Tamura and Nemoto, 2001; Uetake et al., 2002), differently from the monasters formed by centrosomes, where chromosomes locate on the periphery of the asters (Glover et al., 1995; Gonzalez et al., 1998). We believe that the consideration on the nature of the monaster stated above should apply to our 'aster-like structure' as well, i.e. it is unrelated to the premeiotic centrosomes.

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