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Maternal inheritance of centrosomes in mammals? Studies on parthenogenesis and polyspermy in mice

(fertilization/development/microtubules/heredity/mitosis)

GERALD SCHATTEN, CALVIN SIMERLY, AND HEIDE SCHATTEN

Integrated Microscopy Resource for Biomedical Research, University of Wisconsin, 1117 West Johnson Street, Madison, WI 53706

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ABSTRACT **The centrosome, the microtubule-organizing center of the cell, is introduced typically by the sperm at fertilization. In some mammals, however, this paternal pattern of inheritance appears to be violated. The hypothesis that the centrosome is maternally inherited was tested during parthenogenesis, polyspermy, and polygyny as well as after recovery from microtubule inhibition at first mitosis. During parthenogenesis the paternal contribution was absent, and in polyspermy the paternal contribution was multiplied. Haploid and diploid parthenogenotes as well as polyspermic and digynic fertilized eggs each segregated their centrosomes to organize a bipolar mitotic apparatus. Oocytes recovering from a nocodazole block formed two normal bipolar mitotic apparatus; the paternal chromosomes aligned at one spindle equator, while the maternal chromosomes were found at the other. These results show that the centrosome is maternally inherited from cytoplasmic sites in the mouse. The evolutionary switch from paternal to maternal inheritance in mammals might be related to the additional dangers that parthenogenesis represents: a threat to the life of the mother as well as to the life of the fetus.**

The centrosome (1–3) serving as the microtubule-organizing center of the cell defines the cytoskeletal shape. This shape specifies the axis for division, course for locomotion, and direction for polarization. A functional centrosome is typically absent in eggs and is paternally inherited at fertilization in animals ranging from invertebrates such as ctenophores (4), nematodes (5, 6), echinoderms (7, 8), and molluscs (9), through the protochordate ascidians (10), and to lower vertebrates such as fish (11) and amphibians (12, 13). Introduced by the sperm at fertilization, the centrosome typically organizes the radially symmetric sperm aster and, after duplication, organizes the two poles of the first mitotic spindle. This requirement for the sperm centrosome ensures biparental contributions at fertilization and reduces the chances for parthenogenesis. Polyspermy endangers the zygote because of both centrosomal and chromosomal imbalances.

In the mouse, however, unfertilized oocytes have centrosomal foci, as detected with anticentrosome antibodies (8, 14, 15), and these foci, but not sites adjacent to the incorporated sperm nucleus, are used to organize the microtubules found during fertilization (16) and also to form both poles of the first mitotic spindle. These observations have led to the hypothesis that the centrosome is maternally inherited in this mammal (8). Our study uses parthenogenesis, polyspermy, and recovery from microtubule inhibition to test this hypothesis. If the hypothesis is correct, parthenogenotes would be predicted to contain all constituents needed to organize a bipolar mitotic apparatus, which permits division from one cell into two. If paternal contributions are not determinative in this phase of development, then polyspermic oocytes would also be predicted to divide normally.

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MATERIALS AND METHODS

Gamete Collection and Handling. Outbred ICR strain mice (Sprague–Dawley) were used for this investigation. The superovulation, collection of oocytes and embryos after natural matings, and the removal of cumulus and zona were done, as described (16).

Parthenogenetic Activation. Unfertilized oocytes were activated parthenogenetically in 7% ethanol/Hepes-buffered culture medium for 7 min at room temperature (17, 18) and cultured until first mitosis (18 hr after activation) for production of haploid parthenogenotes. Diploid parthenogenotes collected at first mitosis (20–22 hr after activation) were produced by incubating oocytes in 10 μM cytochalasin B for 5 hr to block second polar-body formation after activation.

In vitro fertilization was accomplished by using the methods of Whittingham (19). Zona-intact unfertilized oocytes collected 1–2 hr after the estimated time of ovulation were inseminated with spermatozoa previously capacitated for 1 hr at 37°C in culture medium containing 89 mM NaCl, 4.74 mM KCl, 1.69 mM $CaCl_2$, 1.18 mM $MgSO_4$, 1.18 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.1 mM sodium pyruvate, 5 mM glucose, 24 mM sodium lactate, penicillin G at 100 units per ml, streptomycin sulfate at 5 μg per ml, and bovine serum albumin at 10 mg per ml. Oocytes containing two pronuclei and a second polar body were removed from the insemination medium and cultured until first mitosis (18 hr after insemination) for the production of fertilized diploid eggs. Fertilized digynic zygotes (two-egg nuclei and one-sperm nucleus) were produced by including 10 μM cytochalasin B at insemination and confirming oocytes with three pronuclei, no second polar body, and a single sperm tail (verified later by antitubulin immunofluorescence microscopy).

Polyspermy and Digyny. Polyspermy was achieved by briefly treating unfertilized oocytes with acid culture medium, pH 2.5, to remove the zona pellucida (20) before insemination *in vitro*. Eight hours after insemination, oocytes were collected and embryos exhibiting three or more pronuclei were separated from the clutch of fertilized eggs; embryos with two or more pronuclei were cultured separately to serve as controls. Dispermic-digynic zygotes (two female and two male pronuclei) were produced by including 10 μM cytochalasin B in the insemination protocol; oocytes with four pronuclei, two sperm tails, and no second polar body were judged to be digynic and dispermic. Polyspermic and polygynic embryos were cultured at 37°C until first mitosis, beginning $\approx$22 hr after insemination.

Spindle Formation Around Individual Pronuclei After Recovery from Microtubule Inhibition Before First Mitosis. The role of the maternal centrosome in organizing the first mitotic spindle was investigated after the disruption and subsequent recovery of cytoplasmic microtubules. To perform this experiment, pronucleate-stage oocytes collected from naturally

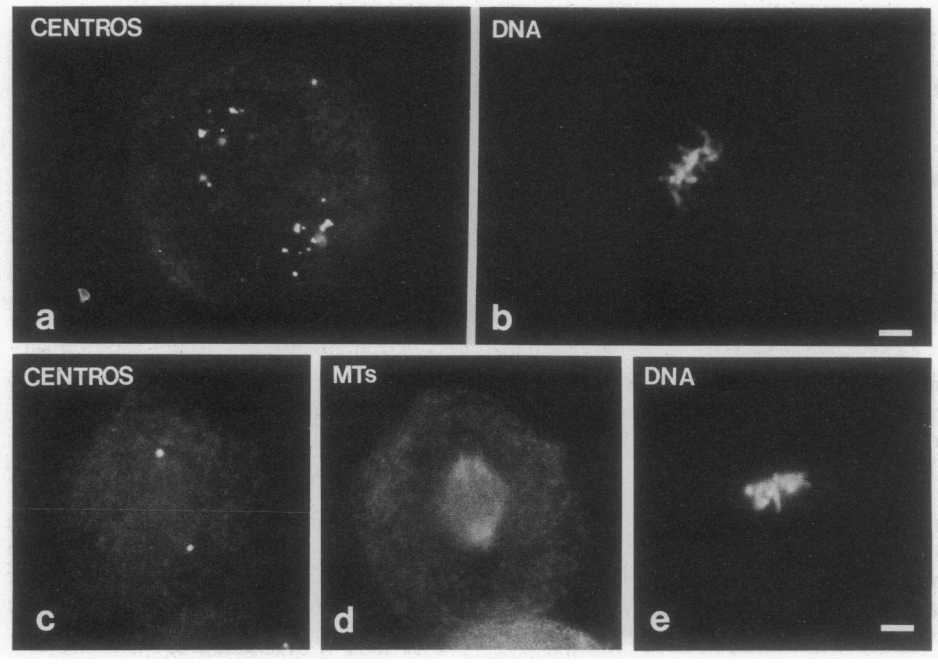

FIG. 1. Centrosomes, microtubules, and chromosome arrangements at first mitosis in haploid and diploid parthenogenotes. (*Upper*) Haploid parthenogenote. At first mitosis, the centrosomal foci partitioned to form two spindle poles (*a*), and the chromosomes aligned along a single metaphase plate (*b*). (*Lower*) Diploid parthenogenote. The ploidy number did not influence the ability of the oocyte to organize a bipolar spindle containing properly positioned centrosomes (*c*), an anastral, barrel-shaped spindle (*d*), and correctly aligned chromosomes on the metaphase equator (*e*). (*a* and *b*) Images were double-labeled for centrosomes and DNA. (*c–e*) Images were triple-labeled for centrosomes, microtubules, and DNA. CENTROS, centrosome detection; MTs, microtubule detection; DNA, DNA fluorescence microscopy. (Bars = 10 μm.)

mated females were blocked before first mitosis by incubation for 12–16 hr in the microtubule-inhibitor nocodazole (5 μM) at 37°C. At the time normal for mitosis, oocytes were washed free of nocodazole, and microtubule recovery was permitted for 1 hr at 37°C before processing for indirect immunofluorescence microscopy.

Centrosome, Microtubule, and DNA Localization. Zona-free oocytes and embryos were permeabilized in a buffer previously shown to stabilize microtubules and centrosomes (8, 16). Fixation was accomplished by using 10 mM dimethyl-3,3′-dithiobispropionimidate dihydrochloride (DTBP; Pierce) prepared in permeabilization buffer, pH 7.9, for 24 hr at room temperature. After fixation, oocytes were rinsed in phosphate-buffered saline/0.1% Triton X-100 detergent.

All oocytes were triple-labeled for centrosomes, microtubules, and DNA. Anticentrosomal and antitubulin antibodies were applied for 40 min at 37°C and followed by a phosphate-buffered saline/Triton rinse of 20 min. Centrosomes were detected by using an autoimmune antiserum (5051) obtained from a patient with scleroderma (14) diluted 1:50 in phosphate-buffered saline; the centrosomes were then labeled with fluorescein-conjugated antihuman secondary antibody. Microtubules were labeled with an affinity-column-purified rabbit antitubulin antibody applied at 50 μg of protein per ml and then with rhodamine-conjugated anti-rabbit antibody. DNA was labeled with 4′,6-diamidino-2-phenylindole (DAPI) at 5 μg/ml in the penultimate phosphate-buffered saline/Triton rinse. Oocytes were mounted in glycerol/1,4-diazobicyclo[2.2.2]octane (DABCO at 100 mg/ml; Aldrich) to retard photobleaching. Epifluorescence microscopy and photography were done as described (8).

RESULTS

Parthenogenesis. Parthenogenetically activated unfertilized oocytes segregate their centrosomes in a manner similar to normally fertilized oocytes during the first cell cycle and organize bipolar mitotic spindles at first division (Fig. 1 *a* and *b*). Unfertilized oocytes parthenogenetically activated in 7% ethanol typically form haploid oocytes with a single pronucleus after extrusion of the second polar body. Centrosomal masses are typically found clustered at the two spindle poles (Fig. 1*a*), and chromosomes are correctly aligned at the spindle equator (Fig. 1*b*) in these eggs. Upon additional culture, haploid parthenogenotes divide to form two equivalent-sized blastomeres.

To determine whether the ploidy state of the parthenogenotes is important in spindle-pole determination, extrusion of the second polar body was blocked by incubating oocytes in 10 μM cytochalasin B for the first 5 hr after ethanol

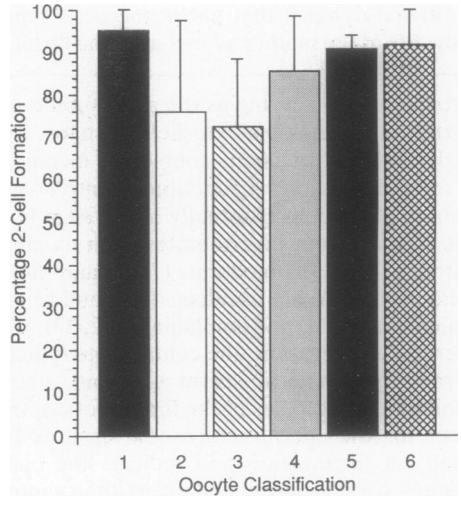

FIG. 2. First cleavage from one cell into two was largely unaffected during parthenogenesis and polyspermy. Bars: 1, fertilized [1N female (♀)/1N male (♂)]—monospermic zona-free oocytes cleaved normally to form two-celled embryos after first mitosis (n = 174); 2, haploid parthenogenote (1N ♀)—parthenogenetic activation of unfertilized oocytes usually resulted in oocytes with a single female pronucleus, and these divided from one to two at high frequency (n = 140); 3, diploid parthenogenotes (2N ♀), created by preventing extrusion of the second polar body with cytochalasin B, also divided from one to two at the time for first cleavage (n = 201); 4, dispermic (1N ♀/2N ♂) zygotes completed mitosis and formed two-celled embryos with equal-sized blastomeres similar to monospermic fertilized oocytes (n = 17); 5, digynic (2N ♀/1N ♂)—tetraploid oocytes, produced by preventing formation of the second polar body (digyny) and by dispermic insemination, also divided from one to two at high frequency (n = 10); 6, digynic/dispermic (2N ♀/2N ♂). Statistical analysis of the reported values were not significantly different between oocyte classes for percent two-cell formation after fertilization or artificial activation ($P < 0.01$; minimum of three trials).

activation. Under these conditions, recovery from the microfilament inhibitor produced an oocyte with two haploid pronuclei from the products of the second meiotic division because the constriction necessary for formation of the second polar body cannot occur. These 2N oocytes enter mitosis ≈20 hr after activation. Anticentrosomal and antitubulin immunofluorescence microscopy demonstrated that these oocytes can correctly organize their centrosomal foci (Fig. 1*c*) into spindle poles, form typical barrel-shaped anastral mitotic spindles (Fig. 1*d*), and align their chromosomes at the first metaphase equator (Fig. 1*e*). These observations suggest that the centrosome is derived from cytoplasmic sites in either haploid or diploid oocytes. Diploid parthenogenotes divided in two at nearly the same frequency seen with fertilized control oocytes (Fig. 2 bar 1, controls; bar 2, haploid parthenogenotes; bar 3, diploid parthenogenotes).

Our results indicate that activated unfertilized oocytes are fully competent to form bipolar mitotic spindles and divide normally, regardless of the ploidy state.

Polyspermy. Polyspermy in most animals is fatal, and the fertilized eggs are unable to form bipolar spindles due to the presence of supernumerary centrosomes. To test the hypothesis that polyspermy might not interfere with first cleavage in the mouse, unfertilized oocytes were treated with acidic medium to remove the zona pellucida, thereby permitting multiple sperm entries. Oocytes with three or more pronuclei were cultured to the time of first mitosis and processed for immunocytochemical detection of centrosomes, microtubules, and DNA. Dispermic (Fig. 3*a–d*) and trispermic mouse

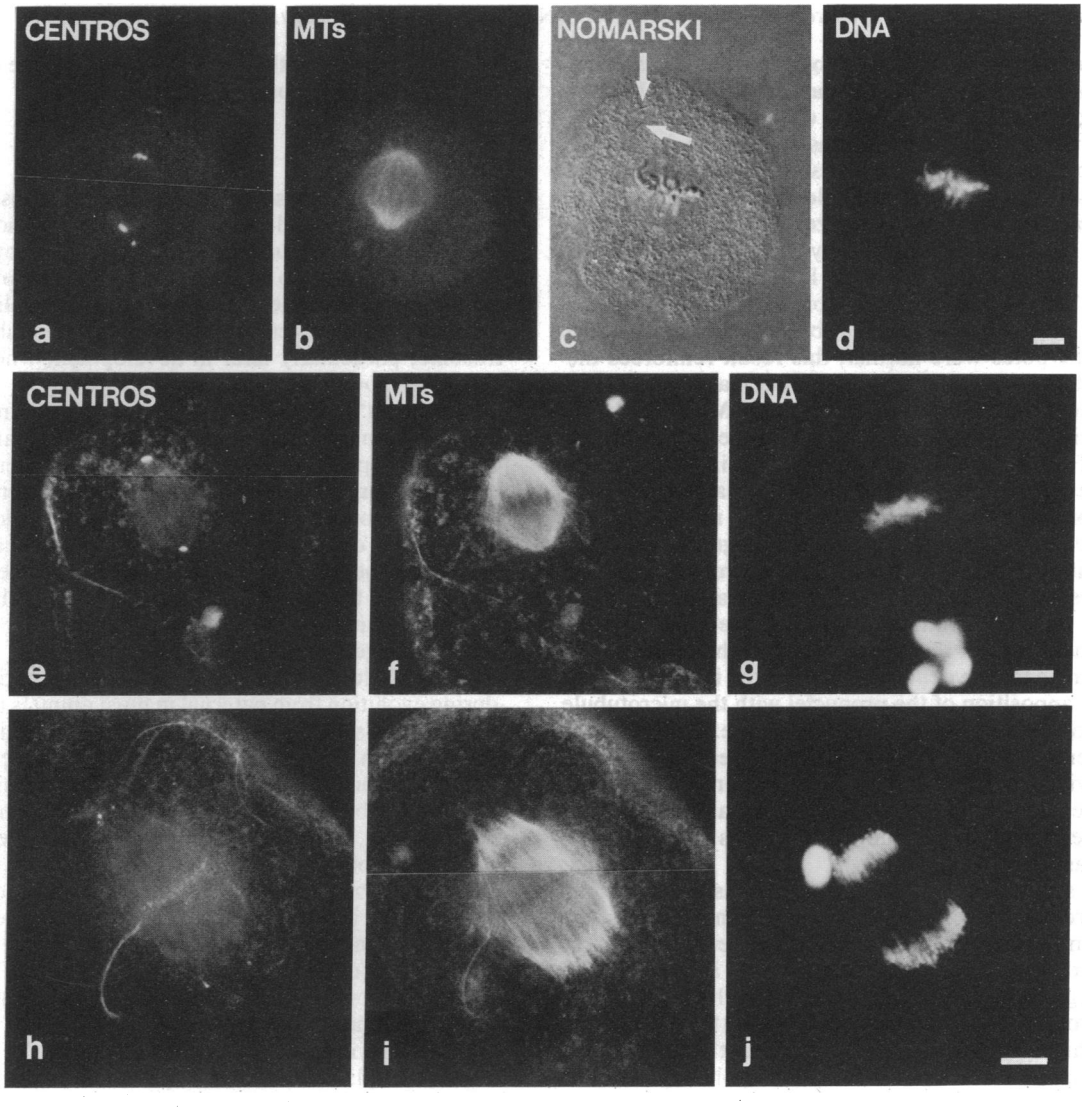

FIG. 3. Centrosomes, microtubules, and chromosome arrangements at first mitosis in dispermic, digynic, and dispermic-digynic fertilized oocytes. (*Top*) Dispermic fertilization. (*Middle*) Digynic fertilization (two egg nuclei with one sperm nucleus). (*Bottom*) Dispermic-digynic fertilization (two egg nuclei and two sperm nuclei). Neither supernumerary sperm nor an extra female pronucleus affects proper centrosomal segregation, bipolar spindle formation, or chromosome alignment on the metaphase plate. At first mitosis, polyspermic oocytes were permeabilized and processed for immunocytochemical detection of centrosomes, microtubules, and DNA. Zona-intact and acid-denuded oocytes were fertilized *in vitro* in the presence of 10 μM cytochalasin B to produce digynic and dispermic-digynic zygotes, respectively. At 6 hr after insemination, oocytes with three pronuclei and a single sperm tail, as verified later with antitubulin immunofluorescence microscopy, were considered as fertilized and digynic zygotes. Oocytes with four pronuclei and two sperm tails were judged to be digynic and dispermic. These oocytes were then cultured at 37°C until first mitosis (22 hr after insemination). In all cases, the centrosomal foci segregated to form two poles (*a*, *e*, and *h*), which organized microtubules into bipolar spindles (*b*, *f*, and *i*), and the chromosomes aligned along a single metaphase (*d* and *g*) or anaphase (*j*) plate. All images were triple-labeled for centrosomes (*a*, *e*, and *h*), microtubules (*b*, *f*, and *i*), and DNA (*d*, *g*, and *j*). (*c*) Nomarski differential-interference contrast optics. CENTROS, centrosome detection; MTs, microtubule detection; DNA, DNA fluorescence microscopy. (Bars = 10 μM.)

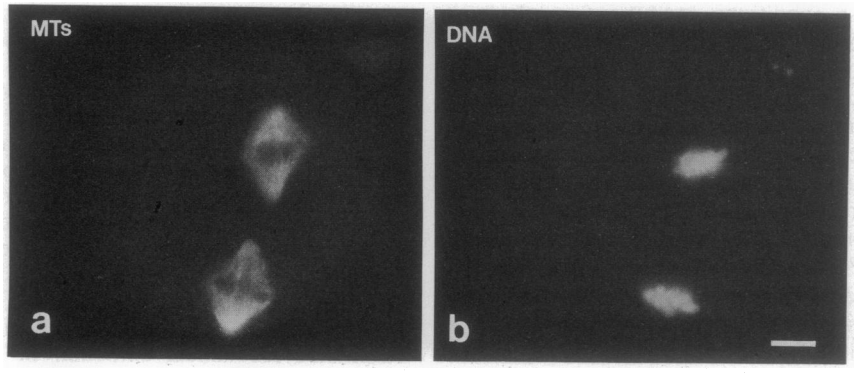

FIG. 4. Oocytes recovering from 5 μM nocodazole-arrest formed two normal bipolar spindles at first mitosis (antitubulin immunofluorescence microscopy). (*a*) The paternal chromosomes aligned at the one spindle equator, while the maternal chromosomes aligned on the other (DNA fluorescence microscopy) (*b*). MTs, microtubule detection; DNA, DNA fluorescence microscopy. (Bars = 10 μm.)

oocytes organized normal bipolar mitotic spindles and cleaved at frequencies comparable to the rate of fertilized control oocytes (Fig. 2 bar 1, controls; bar 4, dispermy). Infrequently the incorporated sperm axoneme was found associated with one of the spindle poles. These results indicate that the sperm is not contributing a dominant spindle pole.

Mitotic spindle formation in fertilized digynic zygotes (Fig. 3*e–g*; Fig. 2 bar 5) and dispermic-digynic zygotes (two sperm nuclei, two egg nuclei; Fig. 3*h–j*; Fig. 2 bar 6) was also unaffected. The additional chromosomes enlarged the metaphase plate, as would be expected, but the number and size of the spindle poles were normal; this result reinforces the conclusion that the centrosome is of maternal origin.

Spindle Reformation After Recovery from Microtubule Inhibition During Pronuclear Migrations. Another way to assess the mode of centrosome introduction at fertilization is to see whether the centrosome preferentially associates with either the male or the female pronucleus after recovery from microtubule inhibition during pronuclear migrations. Pronuclear apposition is sensitive to microtubule inhibitors (15, 16), and the presence of a single bipolar mitotic apparatus associated with the male pronucleus has provided evidence for paternal inheritance of the centrosome in echinoderms (21).

The rationale for the experiments depicted in Fig. 4 was to prevent the apposition of the pronuclei with the microtubule inhibitor nocodazole during first interphase. The separate male and female pronuclei undergo nuclear-envelope breakdown individually, and their respective chromosome sets condense separately. Then the oocytes are washed free of the microtubule inhibitor, and the microtubules of the mitotic apparatus assemble.

Should a single bipolar mitotic apparatus assemble around the male pronucleus, evidence for the paternal introduction of the dominant microtubule-organizing center would be provided; this situation has been shown in invertebrate systems (e.g., refs. 15 and 16). On the other hand, if a single bipolar mitotic apparatus assembles around the female pronucleus, this evidence supports the idea that the female chromatin plays a crucial role in spindle-pole organization. The assemblage of two bipolar spindles around the separate male and female pronuclei would provide evidence for the hypothesis that the centrosome is derived from cytoplasmic maternal sources; this latter alignment is the observed result.

Fig. 4 shows an oocyte treated with 5 μM nocodazole for 15 hr during the pronuclear migrations; the drug was removed, and after an hour the oocyte was processed for microtubule and DNA localization at first mitosis. In Fig. 4*a* two separate bipolar mitotic spindles are organized by maternal cytoplasmic centrosomes, and the maternal and paternal chromosome sets align on the separate spindle equators (Fig. 4*b*). These observations demonstrate that functional centrosomes are not preferentially associated with the male pronucleus; instead, the centrosomes associate equally with both parental chromosome sets.

DISCUSSION

The unfertilized mouse oocyte has all the necessary constituents to organize and replicate spindle poles normally; parthenogenotes divide *in vitro* through the blastocyst stage and have been reported to implant normally (22). However, these results do not completely exclude the possibility that both parents eventually can contribute to the embryonic centrosome, particularly later in development when centrioles appear *de novo* (23).

To suggest that centrosomes are maternally inherited in all mammals is premature. Studies on rabbits (24) and sheep (25) describe the presence of a monastral sperm aster, as might be expected were the centrosome paternally inherited. Dispermic human oocytes from an *in vitro* fertilization clinic (26) divide from one into three, a situation that cannot be explained by the simple inheritance of centrosomes from either the father or the mother. The presence of multiple centrosomal foci in unfertilized oocytes from hamsters (unpublished results) suggests maternal origin.

Why would the mode of centrosome inheritance switch during evolution from paternal in most animals to maternal in mice and possibly other mammals? An arresting theory on the evolution of sexuality involving centrosome competition, which rests on the postulate that the offspring might retain the centrosome best suited for its microtubule organization (27), is supported by the, still controversial (28), report of basal body/centriolar DNA (29). If this theory is correct, then some animals might retain the paternal centrosome, whereas others could inherit this vital structure from the mother, and still others might use a mixture of both sperm- and egg-derived centrosomes. Interestingly, although mitochondria are thought to follow a pattern of strict maternal inheritance, biparental mitochondrial DNA inheritance has recently been found in the mollusc *Mytilus* (30).

Other explanations involve particular aspects of mammalian development: the absence of severe temporal constraints to complete the first cell cycle and the danger to the mother's as well as the embryo's life when aberrant fertilization occurs. In nonmammalian species a premium is placed on rapid development, and the formation of a sperm aster associated with the male pronucleus might speed the events leading to syngamy and accelerate the first cell cycle. In mammals, the first cell cycle is almost a day in length, and the developing embryo must await the proper hormonal triggering necessary for implantation. Consequently there are few demands on the mammalian oocyte to complete fertilization rapidly because the priming of the uterus for implantation takes several days.

Perhaps the evolutionary switch relates to the specific dangers of parthenogenesis to the mammalian mother: She herself might be jeopardized by an aberrant implanted fetus. One mechanism to increase safety is to mandate stringent requirements for biparental fertilization. Although most non-mammalian systems prevent parthenogenesis by requiring the introduction of the sperm centrosome, mammals depend on contributions from both maternal and paternal genomes for development to term (31–33). This switch to genomic, rather than centrosomal, control for ensuring biparental fertilization, might relieve the requirement that the sperm introduce the centrosome. The maternal centrosomes functional during oogenesis could be expected, in some species, to retain their activity during fertilization and embryogenesis, whereas other mammals might rely on a blend of maternal and paternal contributions. Perhaps the lack of any strict requirement for the mode of centrosomal inheritance would permit variability among mammals, a situation that can now be explored.

Note Added in Proof. A report by Sathananthan *et al.* (34) entitled "Centrioles in the beginning of human development" was recently published demonstrating the appearance of centrioles in human oocytes at first mitosis.

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