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Meiosis I in *Xenopus* oocytes is not error-prone despite lacking spindle assembly checkpoint

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The spindle assembly checkpoint, SAC, is a surveillance mechanism to control the onset of anaphase during cell division. SAC prevents anaphase initiation until all chromosome pairs have achieved bipolar attachment and aligned at the metaphase plate of the spindle. In doing so, SAC is thought to be the key mechanism to prevent chromosome nondisjunction in mitosis and meiosis. We have recently demonstrated that *Xenopus* oocyte meiosis lacks SAC control. This prompted the question of whether *Xenopus* oocyte meiosis is particularly error-prone. In this study, we have karyotyped a total of 313 *Xenopus* eggs following in vitro oocyte maturation. We found no hyperploid egg, out of 204 metaphase II eggs with countable chromosome spreads. Therefore, chromosome nondisjunction is very rare during *Xenopus* oocyte meiosis I, despite the lack of SAC.

Introduction

The ability of cells and organisms to maintain genome integrity is essential for cellular and organismal function. Chromosome instability, particularly gain or loss of whole chromosome(s) during mitosis, termed aneuploidy, is the hallmark of most solid tumors. A high degree of chromosome instability, on the other hand, often leads to apoptosis, thus suppressing tumor progression.[4-7] Aneuploid germ cells (eggs or sperms) derived from chromosome segregation errors during meiosis produce aneuploid embryos. With rare exceptions, aneuploid embryos die during various stages of embryonic development. In mice, the only viable aneuploidies are X chromosome monosomy[3] and trisomy 19.[9] In humans, only X monosomy (Turner syndrome) and very few trisomies can be viable, but inevitably carry major birth defects (e.g., trisomy 21 or Down syndrome).[1,4]

Aneuploidies are produced during anaphase of cell division when one or more chromosomes are mis-segregated, resulting in chromosome gain in one daughter cell (hyperploid) and the corresponding loss in another (hypoploid). Given the vital importance of chromosome segregation fidelity, nature has evolved a surveillance mechanism, namely spindle assembly checkpoint (SAC), to control the onset of anaphase during cell division.[3,3] SAC prevents anaphase initiation until all chromosome pairs have achieved bipolar attachment and aligned at the metaphase plate of the spindle. SAC is thought to be activated by "naked kinetochores" (kinetochores unoccupied by microtubules)[39,40] and by the lack of tension between the sister chromatids when both are attached to the same pole (monoploar attachment).[27] In prometaphase, major SAC proteins Mad2 (mitotic arrest deficient 2), Bub1B (budding uninhibited by benzimidazoles 1B), and Bub3 form mitotic checkpoint complex (MCC) at kinetochores. Kinetochore-associated MCC binds and sequesters Cdc20, a key activator of anaphase-promoting complex (APC; an E3 ligase). Cdc20 sequestration at kinetochores prevents APC activation. At metaphase when sister kinetochores are fully occupied by kinetochore microtubules and are bipolar attached, MCC dissociates from kinetochores, releasing Cdc20, which, in turn, activates APC.[33] APC targets cyclin B and securin, among many other protein substrates, for proteolysis.[37] Securin degradation leads to activation of separase and removal of cohesin, thus releasing sister chromatids. Degradation of cyclin B results in the inactivation of cyclin-dependent kinase 1 (CDK1), allowing mitotic exit with ensuing anaphase and cytokinesis.[37]

Meiosis I in animal oocytes is fundamentally different from mitosis of somatic cells. Meiosis I has the unique task of segregating homologous chromosomes. Unlike in somatic cells, where homologous chromosomes are independently segregated, in germ cells, homologous chromosomes are linked due to lengthwise sister chromatid cohesion (by a protein complex named cohesin) and non-sister crossover (homologous recombination), established before birth in mammals. In meiosis I in adult females, the 2 sisters are segregated together (to the first polar body or the mature egg) after the loss of cohesin in chromosome arms. Centromeric cohesin remains until after fertilization, when anaphase II segregates the 2 sisters (to second polar body and the haploid egg

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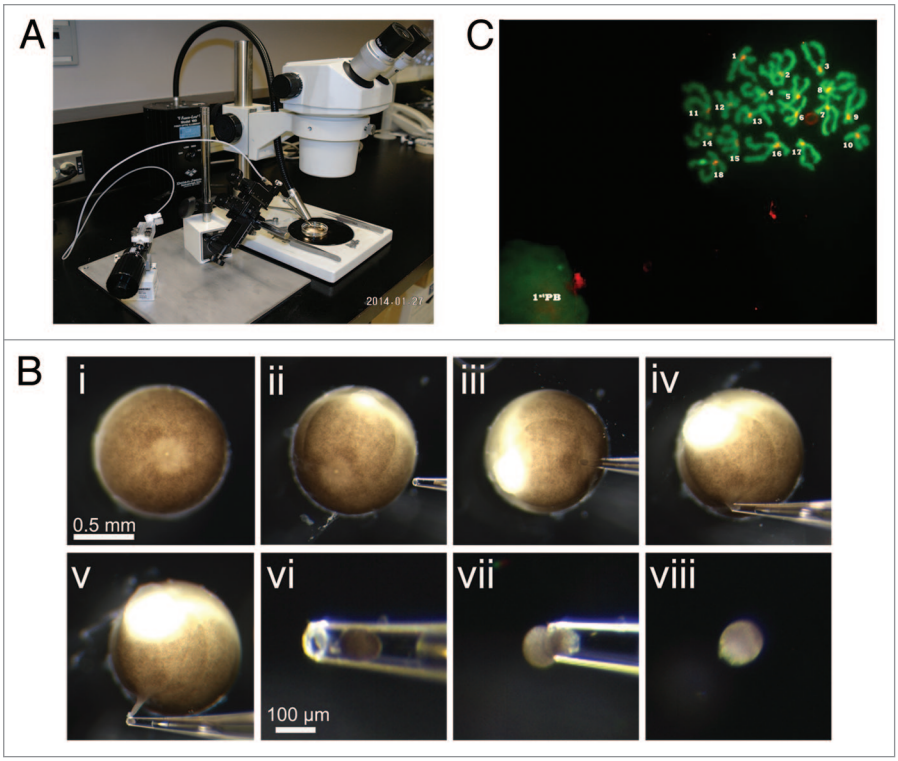

Figure 1. (**A**) Narishige IM-9A microinjector (far left) with a glass needle attached (under the scope and above the dish). (**B**) See text for details. The scale bar in panel i applies to panels i–v, and that in panel vi applies to panels vi–viii. The white patch in some images (iii–v) is an artifact of uneven lighting. (**C**) A typical euploid metaphase II egg with 18 chromosome dyads. The numbers (1–18) are used to facilitate chromosome counting and do not imply chromosome identities. Among the 204 metaphase II spreads, 82 were in the presence of the first polar body (1st PB) chromosomes, suggesting that abscission had not completed in these eggs at the time of karyotyping.[43] DNA in green and Aurora B in red.

respectively).[22] Meiosis I in human oocytes is error-prone primarily due to premature separation of sister chromatids (PSSC) (as appose to whole chromosome non-disjunction)[1,23] caused by premature loss of centromeric cohesin,[8] an error not likely recognized by SAC, which surveys kinetochore–microtubule interaction and tension.[33] In addition, animal oocytes lack centrosomes and assemble a bipolar spindle from multiple microtubule-organizing centers;[11,41,44] in acentrosomal spindles, kinetochore microtubules are entirely absent in some species[45,48] or, in other species, are relatively scarce and only discernable after experimental destruction of non-kinetochore microtubules.[2,6,12,18,20,36,44] Finally, the essence of SAC is the ability of the spindle to control the biochemistry of cytoplasmic proteins (Cdc20, cyclin B, securin, etc.). It seems that the mechanism would be quite different in somatic cells and large animal eggs, where the spindles are similar in size but the cytoplasm volumes are different by thousands- to millions-fold.

It is therefore not surprising that the presence of SAC and its function in animal eggs has been controversial.[29] Complete disruption of spindle microtubules by nocodazole in mouse oocytes causes reversible metaphase I arrest, suggesting the presence of a functional SAC.[2] Moreover, deficiency of SAC proteins increases mouse egg aneuploidies in vivo[12,24,31] and in vitro.[17,26] On the other hand, incorporating chromosome monovalents into meiosis I spindle causes obligatory monopolar attachment, and yet mouse oocytes with many misaligned chromosomes proceed to anaphase and polar body emission without delay.[4,25,34,42] Finally, older mice exhibit significantly higher egg aneuploidy rates due to age-related cohesin loss[5,16,28,38] and the consequent PSSC during meiosis I or meiosis II,[5,46,49] with no evidence that SAC function in these oocytes are compromised.[7,28]

In contrast to mouse oocytes, complete disruption of microtubules by nocodazole in *Xenopus* oocytes does not cause metaphase I arrest. Similarly, monopolar spindle, which causes SAC-mediated metaphase arrest in mitotic cells, does not cause metaphase I arrest in frog oocytes.[43] In this study, we have asked the question whether *Xenopus* meiosis I is error-prone, given the lack of SAC.

Results and Discussion

The classical chromosome spread method suitable for mammalian eggs[10] is not directly applicable to the much larger *Xenopus* eggs. We have recently developed a karyotyping method that has enabled us to analyze chromosome morphology during *Xenopus* oocyte meiosis.[43] In this method, we first excise a mini-cell containing the meiotic spindle including all chromosomes, reducing the cytoplasmic volume by >5000-fold (**Fig. 1A and B**). We then subject the mini-cell to chromosome spread. This method produces intact meiotic chromosome arrays, with chromosome-associated proteins, such as the centromere-bound Aurora B, at all stages of meiosis: 18 bivalents at prometaphase I and metaphase I, 2 sets of 18 dyads (monovalents) at anaphase I, 18 dyads at metaphase II with or without the partially de-condensed first polar body chromosomes, and, following parthenogenetic activation, 2 sets of sister chromatids (without Aurora B) at anaphase II.[43]

We performed karyotype analyses of *Xenopus* eggs following in vitro oocyte maturation, shortly after first polar body emission, when individual metaphase II chromosomes have relatively short arms and are therefore easily identified.[43] In 15 experiments using 10 females between 14–32 months of age, we subjected 313 eggs to the karyotype procedure. Of the 256 spreads which contained chromosomes, we obtained 204 countable metaphase II spreads, summarized in **Table 1**. The majority (140/204) had a euploid karyotype, with 18 chromosome dyads (**Fig. 1C**). The next most abundant karyotype is of 17 dyads (29/204). The remaining 34 have various numbers of dyads, from 16 to less than 12. Most significantly, we did not find any hyperploid eggs (19 or more dyads). Only one egg (1/204) was

Table 1. Karyotypes of in vitro-matured *Xenopus laevis* eggs

Karyotype	MI	AI	Uncertain	18 (Euploid)	17	16	15	14	13	≤12	PSSC (17.5)	Hyperploid
# of eggs	2	7	43	140	29	13	7	6	3	5	1	0
					63							
				204								
Total	256											

found to contain an unpaired sister (plus 17 dyads; 17.5). Only 2 eggs (both from the same batch of oocytes) were found in metaphase I with 18 intact bivalent chromosomes. In addition, 7 eggs (all 7 from the same batch of oocytes) contained chromosomes at anaphase I.[43] The remaining 43 eggs were at metaphase II, but the chromosomes were not spread well enough to be counted accurately.

Finding 0 hyperploid metaphase II eggs in 204 countable metaphase II spreads indicates that the maximal frequency of hyperploid eggs, with 95% confidence, is less than 2%. The occurrence of "hypoploid" eggs (≤17 dyads) was likely the result of technical loss of chromosomes during the procedure.[21] The ratio of euploid karyotypes over "hypoploid" karyotypes is very similar to those reported in experiments on mice,[21,46] indicating that our method for karyotyping *Xenopus* eggs is as efficient as that for mouse eggs. These results therefore indicate that chromosome nondisjunction (i.e., hyperploidy) in *Xenopus* oocyte meiosis I is very rare. The rare occurrence (1/204) of unpaired sister chromatids in metaphase II *Xenopus* eggs suggest that the time lapse (8–26 mo) from sexual maturity (6-mo-of-age[19]) had little effect on the occurrence of PSSC, in contrast to the significant aging-dependent increase of PSSC found in mice.[21,46] While it is thought by most that mammalian females are born with a finite number of prophase-arrested oocytes, fish and amphibian ovaries may contain germ line stem cells capable of replenishing the oocyte pool after every spawning.[19,35]

The chromosome nondisjunction rate (hyperploidy) in *Xenopus* meiosis I, as reported here, is no greater than that in oocyte meiosis I in young mice,[21,46] suggesting that the lack of SAC in *Xenopus* oocyte meiosis does not render it error-prone. Like meiosis in *Xenopus* oocytes, early mitoses in *Xenopus* embryos similarly lack checkpoint control.[13,32] These embryonic mitoses are rapid (every 30 min in *Xenopus*, but even faster in other organisms, such as *Drosophila*). One might argue that the lack of SAC in early embryos of these species is necessary for the rapid and synchronous cleavage divisions, since a checkpoint delay in some cells will disrupt the synchronicity with deadly outcome. Speedy embryonic development is clearly important for these immobile and unprotected embryos. But is chromosome segregation fidelity compromised in these early mitoses? This remains to be determined.

Materials and Methods

Sexually mature and oocyte-positive *Xenopus* laevis females (10–12 mo of age) were purchased from Nasco and maintained in XenopLus Housing System (Tecniplast), with water temperature set at 19 °C. For this project, the frogs were used 4–20 mo after arrival (14–32 mo of age). Each female was primed with 100 IU PMSG (Sigma-Aldrich) and sacrificed 3–10 d after hormone injection. Oocytes were isolated by manual defolliculation[30] and kept at 18 °C in oocyte cultural medium (OCM; 60% of L-15 medium [Sigma-Aldrich], supplemented with 1.07 g BSA per liter, mixed with 40% autoclaved water, and 50 μg/ml gentamicin [Gibco]). Oocytes were incubated in OCM with 1 μM progesterone and monitored for germinal vesicle breakdown (GVBD; indicated by the appearance of a depigmented spot at the animal pole) every 10 min. GVBD oocytes were transferred individually into fresh OCM without progesterone.

Two and half (2.5) hours after GVBD, oocytes were transferred to OR2 medium (83 mM NaCl, 2.5 mM KCl, 1 mM Na_2HPO_4, and 5 mM Hepes, pH 7.8) containing 10 μg/ml cytochalasin B and 1 mg/ml BSA. After 5 min incubation, the vitelline membranes (depicting as fuzzy egg outline) were torn off partially, using 2 pairs of fine forceps, at the animal pole to expose the maturation spot (**Fig. 1B**, ii). A glass needle of ~70 μm inner diameter at the tip (prepared as described below) attached to an IM-9A microinjector (Narishige; **Fig. 1A**), was placed over the plasma membrane at the spindle anchoring site, evident as a translucent spot at the center of a larger depigmented maturation spot (**Fig. 1B**, i and ii). A negative pressure was applied manually through the Narishige injector to aspirate the translucent spot into the needle (**Fig. 1B**, iii and iv). The oocyte was moved away slowly (**Fig. 1B**, v), severing a mini-cell inside the needle (**Fig. 1B**, vi). The mini-cell was immediately expelled by applying a positive pressure (**Fig. 1B**, vii and viii). Mini-cells were immediately transferred into water (as hypotonic solution) containing 1 mg/ml BSA. Ten (10) minutes later, the mini-cells were transferred individually, with minimum solution, onto a glass slide pre-wet with fixative (1% paraformaldehyde in water, containing 0.15% Triton X-100 and 3 mM dithiothreitol, pH 9.2).[15] The slides were kept in humid box for overnight before air drying for 1 h. The slides were rinsed in 0.5% photoflo (Kodak) in water for 1 min and then rinsed 3 times with PBS and subjected to immunostaining with antibodies against Aurora B[50] and sealed with mounting solution containing 1.5 μg/ml 4', 6-diamidino-2-phenylindole (DAPI) for DNA counterstaining. The slides were imaged in a Zeiss Axiovert 100 scope with a 63× oil objective. The images were pseudo-colored using Volocity (Improvision), followed by karyotype determination.

The needles were made by pulling the glass micropipettes (30 μL Microcaps, Drummond Scientific Company) with Micropipette Puller (Model P-97, Sutter Instrument Co; Settings:

P[ressure] = 300, Heat = 494, Pull = 200, Vel[ocity] = 60, Time = 200). The pulled needles were cut to get 70 μm inner diameter at the tip before heat-polished using Microforge Microscop (Model: MF-9; Narishige).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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