Current Biology

Report

Intrinsically Defective Microtubule Dynamics Contribute to Age-Related Chromosome Segregation Errors in Mouse Oocyte Meiosis-I

Highlights

- Microtubule dynamics is altered in oocytes from naturally aged mice
- Multipolar spindles precede missegregation of intact sister chromatid pairs
- Chromosome swapping reveals spindle defects are not attributable to chromosome aging

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In Brief

Why chromosomes frequently missegregate in oocytes from aging females is poorly understood. Here, using live imaging and micromanipulation approaches, Nakagawa and FitzHarris show that oocytes from older mice exhibit altered spindle microtubule dynamics, providing a novel explanation for missegregation of intact sister chromatid pairs in meiosis-I.

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Intrinsically Defective Microtubule Dynamics Contribute to Age-Related Chromosome Segregation Errors in Mouse Oocyte Meiosis-I

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SUMMARY

Chromosome segregation errors in mammalian oocytes compromise development and are particularly prevalent in older females, but the aging-related cellular changes that promote segregation errors remain unclear [1, 2]. Aging causes a loss of meiotic chromosome cohesion, which can explain premature disjunction of sister chromatids [3–7], but why intact sister pairs should missegregate in meiosis-I (termed non-disjunction) remains unknown. Here, we show that oocytes from naturally aged mice exhibit substantially altered spindle microtubule dynamics, resulting in transiently multipolar spindles that predispose the oocytes to kinetochore-microtubule attachment defects and missegregation of intact sister chromatid pairs. Using classical micromanipulation approaches, including reciprocally transferring nuclei between young and aged oocytes, we show that altered microtubule dynamics are not attributable to age-related chromatin changes. We therefore report that altered microtubule dynamics is a novel primary lesion contributing to age-related oocyte segregation errors. We propose that, whereas cohesion loss can explain premature sister separation, classical non-disjunction is instead explained by altered microtubule dynamics, leading to aberrant spindle assembly.

RESULTS AND DISCUSSION

Accurate chromosome segregation is accomplished by correct attachment of spindle microtubules (MTs) to kinetochores assembled on centromeric DNA. Whereas mitosis separates sister chromatids, meiosis-I separates homologous chromosomes, sister kinetochores forming a single microtubule-binding unit, allowing attached sister pairs to be pulled poleward in anaphase (see Figure S1). In human oocyte meiosis, the likelihood of segregation errors increases with maternal age. Aneuploidy is relatively common in young women but increases further in the 30s, and this increase is considered a leading cause of age-related infertility [1, 2]. Mouse oocytes also experience a dramatic increase in chromosome segregation errors, from <5% in oocytes aged 3 months to 30%–50% of oocytes in mice aged over 12 months [4, 8, 9]. Whereas this is a substantial difference in time course compared to humans, mouse presents the best available model for understanding the mechanistic impacts of aging upon oocyte chromosome segregation. Mouse and human oocytes both exhibit two broad categories of segregation error in meiosis-I: gains and losses of intact sister pairs (termed "non-disjunction") and the emergence of prematurely separated sister chromatids (PSSCs), the molecular explanations for both of which have long been mysterious [3, 4, 9–11] (summarized in Figure S1). Recent evidence from mouse and human oocytes suggests that age-related PSSCs may be underpinned by a progressive loss of the cohesin complexes that maintain sister chromosome and kinetochore unity in meiosis-I oocytes [4–7]. However, because it is difficult to experimentally manipulate cohesin levels in mammalian oocytes [12], directly determining whether cohesin loss is responsible for all segregation errors is technically challenging. In addition, non-disjunction in meiosis-I is not obviously explained by cohesion loss. To rationalize meiosis-I non-disjunction with cohesion loss, it has been hypothesized that altered kinetochore geometry that is evident when sister chromatids experience cohesion loss [4, 5] might render kinetochores more prone to microtubule misattachments [1, 4, 13]. However, kinetochore-MT misattachments in meiosis-I in oocytes from aged mice correlate poorly with cohesion loss [14], as do lagging anaphase chromosomes [15]. Thus, cohesion loss may not explain non-disjunction and is therefore likely not the only aging-related cellular change driving chromosome segregation errors in oocytes. Consistent with this notion, classic studies of human oocytes observed defective spindle morphology in oocytes from older women, alluding to a possible impact of age upon microtubules [16, 17]. However, the dynamics of spindle assembly in oocytes from older females, and how altered microtubule behavior might impact chromosome segregation, is yet to be explored.

To probe how aging affects oocyte meiosis-I microtubule dynamics, we performed live confocal time-lapse imaging of spindle assembly in 6- to 12-week-old ("young oocytes") and ~60-week-old mice ("aged oocytes") in a contemporaneous side-by-side manner (Figure 1A). The vast majority of young oocytes (90.3%) formed a normal bipolar spindle within 3 hr of meiosis resumption, as indicated by loss of the meiotic prophase

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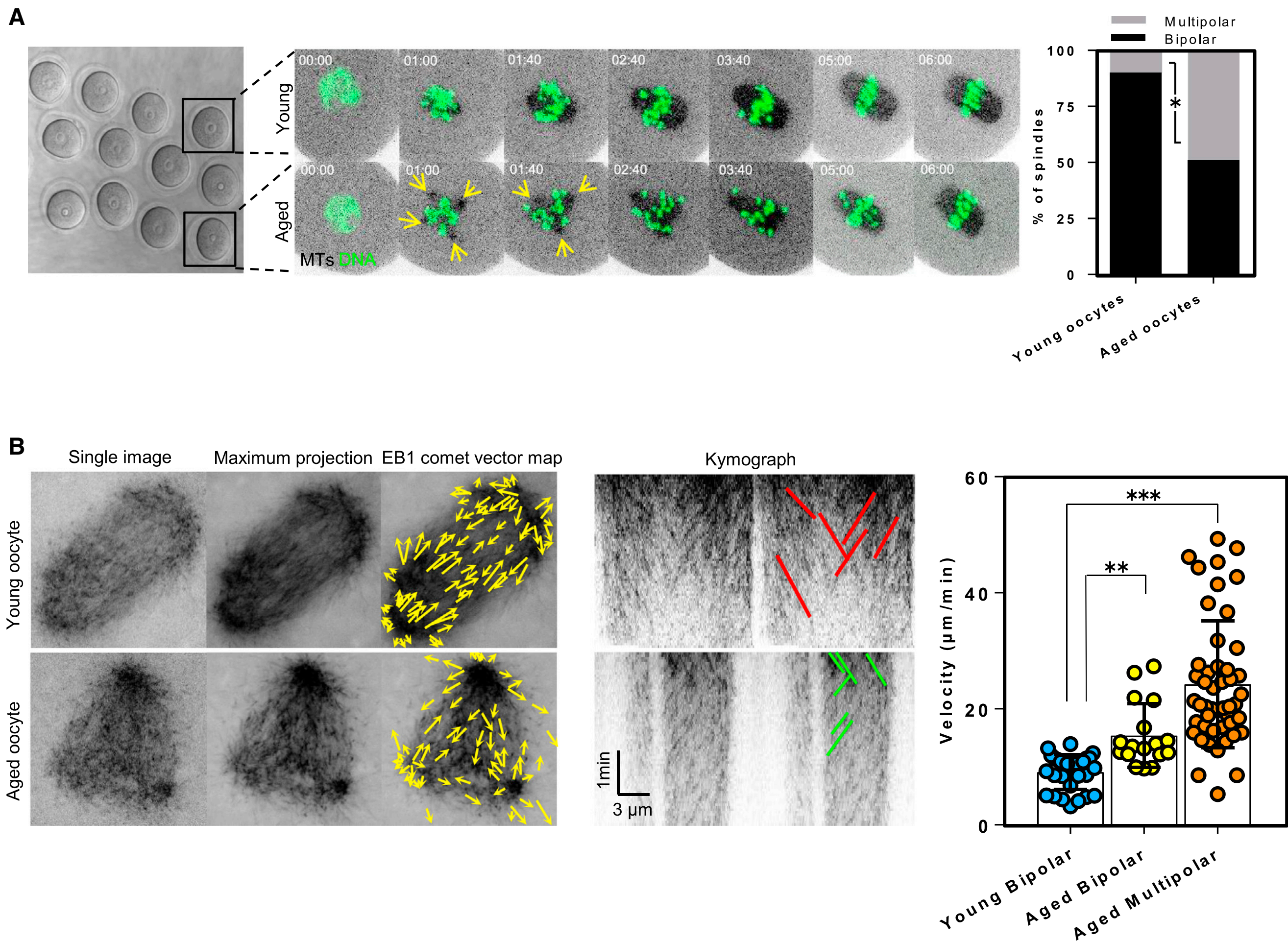

Figure 1. Chaotic MT Dynamics in Aged Oocytes

(A) Spindle assembly was observed in young and aged oocytes side by side using EB1:EGFP (gray) and H2B:RFP (green). The chart to the right represents quantification of multipolar spindle formation in young (n = 31) and aged (n = 35) oocytes. Note that significantly more multipolar spindles were formed in aged oocytes (CHI2; $p < 0.01$).

(B) Rapid acquisition (2.578 s^{-1}) confocal fluorescence time-lapse imaging of EB1:EGFP was performed to analyze the directionality and velocity of microtubule growth events. EB1:EGFP "comets" seen in the maximum projection image indicate individual MT growth events. Yellow arrows overlaid upon the maximum time-projection image indicate the directionality of individual MT growth events identified manually in movies. Note that MT growth events emanate from spindle poles. Kymographs in the middle panel were used to analyze growth event velocities. Greater than or equal to four tracks were selected randomly for analysis from ten young oocytes, five aged oocytes with bipolar spindles, and seven aged oocytes with multipolar spindles. Note that growth velocities are greater in aged oocytes, particularly those with multipolar spindles. **$p < 0.05$; ***$p < 0.01$.

Error bars indicate SEM. See also Figures S1 and S2.

nucleus (germinal vesicle breakdown [GVBD]), and aligned chromosomes by 5 hr (Figure 1A), as anticipated [18, 19]. Strikingly, however, ~50% of aged oocytes developed spindles with more than two spindle poles 1–3 hr after GVBD (Figure 1A). The multipolar spindles resolved into normal bipolar structures in all cases by 5 hr post-GVBD (Figure 1A). To understand the architecture of the multipolar spindles, we first immunolabeled the MT-organizing center component pericentrin, finding it to be highly enriched at the multiple spindle poles in aged oocytes (Figure S2A). Second, for detailed examination of microtubule layout in multipolar spindles, we analyzed microtubule plus-end growth events using rapid acquisition imaging of EB1:EGFP [20]. EB1:EGFP comets emanated from the multiple spindle poles in aged oocytes (Figure 1B), consistent with the multiple poles each being major sites of MT generation. Quantitative kymograph analysis [21] revealed that spindle MT growth velocities were relatively uniform in young oocytes (9.11 μm/min) but far more variable with increased mean growth velocity in aged oocytes, especially within multipolar spindles (24.25 μm/min; $p < 0.01$; Figure 1B). Total spindle MT mass was not different between young and aged oocytes, suggesting that differences in growth velocities were not due to broad changes in MT assembly dynamics (Figure S2B). Third, we used cold-shock analysis to examine the layout of stable microtubules. Whereas cold-stable microtubules were arranged in the predicted stereotypic spindle arrangement in bipolar spindles (Figure S2C), multipolar spindles in aged oocytes exhibited fewer stabilized microtubules ($p < 0.01$), and those that were observed were chaotically

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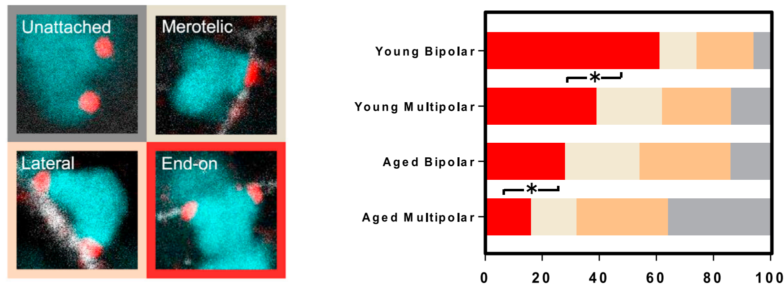

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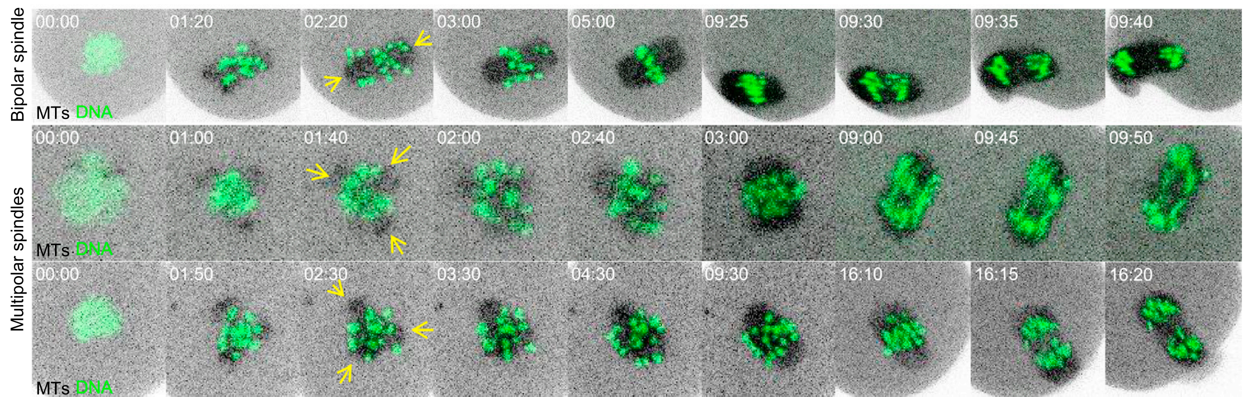

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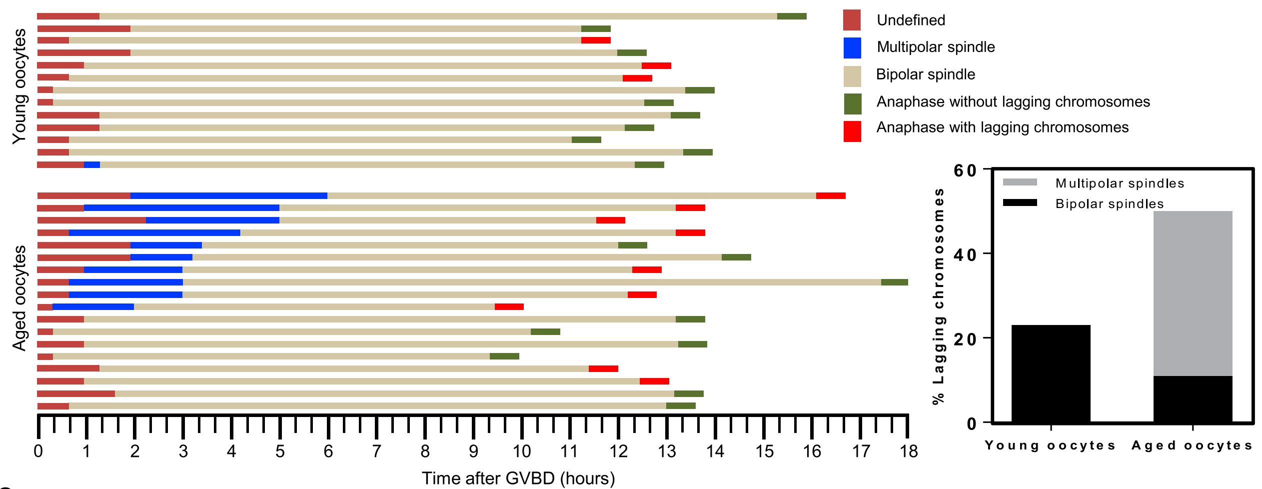

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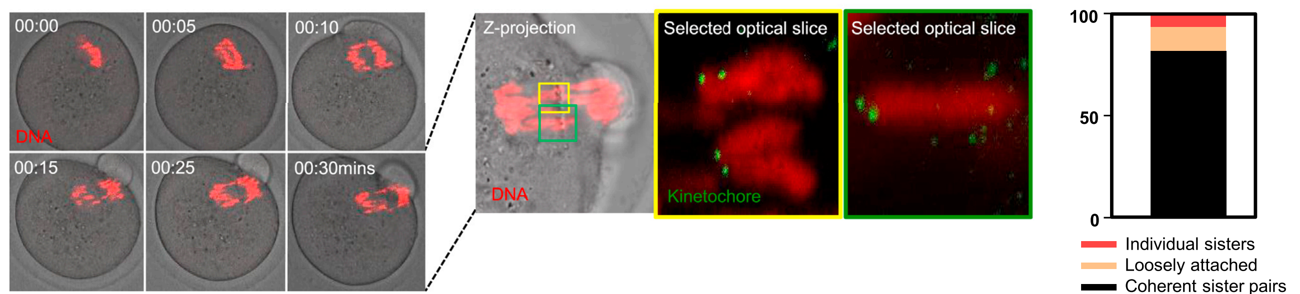

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arranged (Figure S2C). Thus, maternal aging severely impacts microtubule growth and organization in oocytes, such that approximately half of oocytes from older females exhibit transient multipolar spindles with chaotic microtubule architecture and dynamics.

Transient Multipolar Spindles Cause Missegregation of Intact Sister Pairs

We next wondered whether multipolar spindle formation might contribute to segregation errors in oocyte meiosis-I. Elegant recent work showed that transient multipolar spindles in cancer cells lead to chromosome segregation errors by promoting MT-kinetochore attachment defects, which cause lagging chromosomes in anaphase [22, 23]. We therefore live-imaged spindle formation to individually identify oocytes with spindles that had progressed through a multipolar phase and then examined kinetochore-MT attachment using cold-shock assays in mid-late meiosis-I (7 hr after GVBD). Oocytes that had passed through a multipolar phase had a substantially lower number of correctly attached kinetochores (28.0% versus 16.5% in aged oocytes with bipolar and multipolar spindles, respectively; $p < 0.04$; Figure 2A) and, consistent with the reduced number of stable microtubules observed earlier in meiosis (Figure S2C), had substantially more unattached kinetochores (14.1% and 36.2% in aged with bipolar and multipolar spindles, respectively; Figure 2A). Next, we used live imaging throughout meiosis-I to analyze chromosome alignment and segregation. Whereas alignment was complete by 5 hr after GVBD in 47% of aged oocytes that assembled a bipolar spindle without progressing through a multipolar phase, only 7.2% of oocytes that had passed through a multipolar spindle phase had aligned chromosomes (Figure S2D; $p < 0.05$). Thus, although chromosomes did eventually align correctly in the vast majority of old oocytes once spindles became bipolar, transient multipolar spindles were associated with retarded chromosome alignment (Figure S2D). Next, we analyzed anaphase chromosome segregation behavior, specifically quantifying lagging anaphase chromosomes, which are indicative of an increased likelihood of chromosome missegregation and aneuploidy [4, 24, 25]. Lagging anaphase chromosomes were observed in only 23% of young oocytes but in 50% of aged oocytes (Figure 2B; $p < 0.05$), similar to previous reports [4, 5, 26]. Strikingly, aged oocytes without a multipolar spindle exhibited lagging chromosomes only 25% of the time, similar to young oocytes, whereas aged oocytes with multipolar spindles exhibited anaphase lagging chromosomes 70% of the time (Figure 2B; $p < 0.03$). Thus, the increase in anaphase lagging coincides with the occurrence of a multipolar spindle phase.

We next wanted to determine whether the lagging anaphase chromosomes in meiosis-I in aged oocytes are coherent sister pairs or prematurely individualized sisters. To do this, we performed live imaging of meiosis-I and fixed oocytes in mid-anaphase for higher resolution immunofluorescence analysis of chromosome structure. Strikingly, lagging anaphase chromosomes were sister pairs possessing two distinct kinetochores in 94% of cases (Figure 2B). In some cases, the sisters appeared loosely attached, and in just one case (6%), the laggards observed in live imaging appeared to be two individual sisters (Figure S3), consistent with cohesion loss [4, 5, 7]. Thus, lagging chromosomes that arise in meiosis-I in aged oocytes with multipolar spindles are usually intact sister pairs. Together, these experiments suggest that multipolar spindles are the prelude to chromosome non-disjunction in aged oocytes.

Multipolar Spindle Formation in Aged Oocytes Is Caused by Altered Microtubule Dynamics, Not Chromosome Changes

In somatic cells, conditions that alter kinetochore size or shape affect the likelihood of aberrant microtubule attachment and chromosome segregation errors [27, 28]. Thus, given that aging affects sister chromosome cohesion and therefore sister-kinetochore spacing [4, 5, 9], it was hard to exclude the possibility that the differences in spindle behavior we had observed might be a secondary effect of chromosome aging. We therefore designed experiments to discriminate the effects of chromosome aging from microtubule dynamics.

First, we sought to analyze microtubule behavior in young and aged oocytes in the complete absence of chromosomes (Figure 3Ai). In mouse oocytes, removal of the prophase nucleus (germinal vesicle) triggers the formation of chromosome-free spindle-like structures with well-defined focused poles [29], which we herein term "pseudospindles". We therefore compared pseudospindle assembly in young and aged oocytes.

Figure 2. Transient Multipolar Spindles Cause Meiotic Errors

(A) Passage through a multipolar intermediate stage decreases end-on attachment. Representative examples of MTs attachment to kinetochores are shown. Kinetochore-MT attachments were classified into four categories: "unattached" (gray); "merotelic" (light green); "lateral" (beige); and "end-on" (red). Analysis of kinetochore (K)-MTs attachment status in young (n = 15) and aged (n = 17) oocytes is shown. End-on attachments were significantly fewer when multipolar spindles had been observed. This holds true both in young and aged oocytes. More unattached kinetochores were observed in oocytes that had progressed through a multipolar phase.

(B) Analysis of the relationship between multipolar spindles and lagging chromosomes. Spindle formation and chromosome segregation were imaged from germinal vesicle breakdown (GVBD) until the first polar body (PB) extrusion. The images in (Bi) show one example of a bipolar spindle resulting in no lagging and two examples of multipolar spindles resulting in lagging. (Bii) presents longitudinal time course plots of all oocytes examined (young oocytes n = 13; aged oocytes n = 18). The bar chart compares proportions of lagging chromosomes corresponding to bipolar and multipolar spindles in young and aged oocytes. Note that lagging corresponds closely with multipolar spindle intermediates.

(C) Experiment to determine whether lagging chromosomes observed by live imaging in aged oocytes are coherent sister pairs or individualized sister chromatids. Oocytes were imaged live and immediately fixed when a lagging anaphase was observed for kinetochore examination. Representative images of lagging chromosomes in a live oocyte that is expressing H2B:RFP are shown (left). The inset shows the same oocyte having been fixed, immunolabeled for kinetochore identification with CREST, and re-imaged at higher resolution. Note that the bright field H2B:RFP overlay is a maximum intensity projection to show all chromosomes, whereas the zoomed images are optical slices to allow clear visualization of kinetochores. The chart quantifies the observation, showing that anaphase lagging chromosomes were intact sister pairs in 16/17 cases observed (94%).

See also Figures S1 and S3.

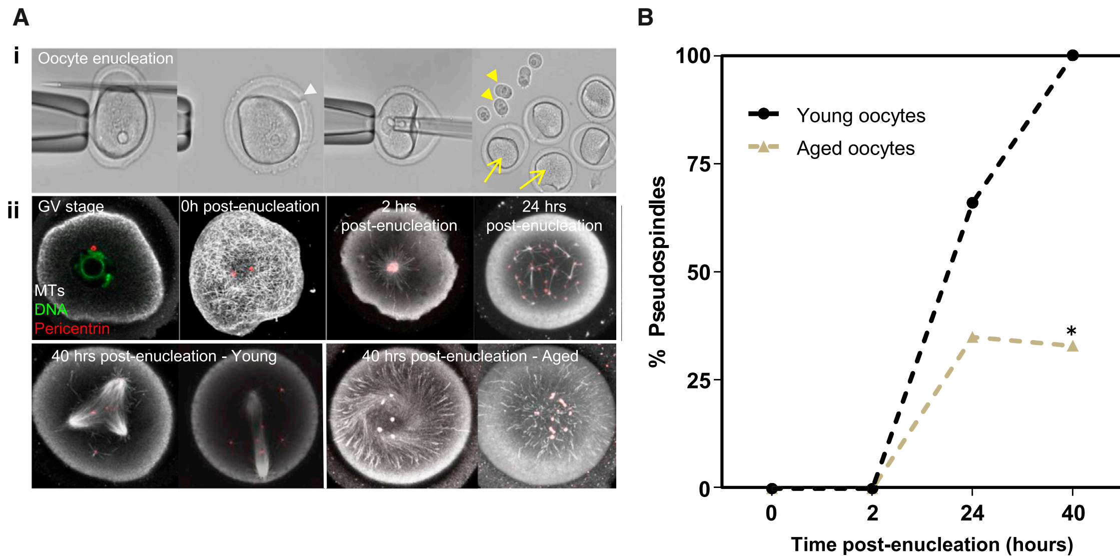

Figure 3. Distinct MT Behavior in Enucleated Young and Aged Oocytes

(Ai) Example of the oocyte enucleation approach used to examine MT behavior in the absence of chromosomes. The zona pellucida was first cut with a glass needle (left panel). An enucleation pipette was then inserted via the resulting slit (white arrowhead). The enucleated oocytes (right panel, yellow arrow) were then used for further experiments. The removed nuclei are indicated by yellow arrowheads.

(Aii) Representative examples of pseudospindle formation. Enucleated oocytes were fixed at different time points (0–40 hr after enucleation) and then labeled for pericentrin (red), microtubules (gray), and DNA (green).

(B) Analysis of pseudospindle formation in young and aged oocytes. Note that all enucleated young oocytes examined at 40 hr exhibited clear pseudospindles (n = 11). In contrast, only 33% of enucleated aged oocytes exhibited pseudospindles (n = 9; CHI2; $p < 0.01$).

Both in young and aged oocytes, enucleated oocytes assembled multiple cytoplasmic microtubule-organizing centers (MTOCs) around 2 hr after initiation of oocyte maturation (Figure 3Aii). In young enucleated oocytes, the MTOCs were replaced by pseudospindles by 40 hr after enucleation in all cases. However, pseudospindles formed in only 33% of aged oocytes (Figure 3B; $p < 0.01$). Thus, maternal aging impacts oocyte microtubule behavior, entirely independent of any aging impact upon the chromosomes, as evidenced by a reduced propensity to form pseudospindles.

Second, to determine directly whether multipolar spindles are caused by aging of the nuclear contents, we used enucleation and electrofusion to perform reciprocal nucleus transfer between young and aged oocytes—thereby creating aged oocytes containing the nucleus from a young oocyte and, conversely, young oocytes with the nucleus of an aged oocyte (Figure 4A). Spindle formation and chromosome alignment were then examined in both situations in a side-by-side manner using live imaging as previously. Transient multipolar spindle formation was rare in young oocytes containing aged chromosomes (11% multipolar spindles; Figure 4B). In contrast, strikingly, multipolar spindles were common in aged oocytes with young chromosomes (75% multipolar spindles; Figure 4B; $p < 0.01$). Consistent with the previous results, multipolar spindles exhibited retarded chromosome alignment (Figure S4). Thus, the effects of age upon microtubule dynamics arise independently of impacts upon the nuclear material.

Multifactorial Age-Related Oocyte Aneuploidy

Together, our experiments define an age-induced defect in spindle assembly that predisposes oocytes to chromosome segregation defects, which is not attributable to age-related cohesion loss. Importantly, our data do not contradict the notion that age-related cohesion loss participates in oocyte aneuploidy. Rather, whereas cohesion loss is the precursor of premature sister chromatid disjunction, our data suggest that classical non-disjunction is a result of faulty spindle dynamics. A distinct etiology of the two types of error likely explains the lack of correlation between cohesion loss and misattachment and lagging previously reported [14, 15]. In human oocytes, meiotic recombination events also affect the likelihood of segregation error, with reduced or altered recombination linked to non-disjunction and trisomy [10, 30]. Our data thus reinforce the notion that mammalian oocyte aneuploidy is multifactorial.

Multipolar spindles preceding lagging chromosomes had previously been observed in some human oocytes [31], but whether this related to age of the oocyte donors or the heterogeneity of human research oocytes was unknown. Our data using a tightly controlled mouse model suggest that multipolar spindles are indeed a feature of aging that contribute to oocyte aneuploidy and are an inherent behavior of microtubules rather than attributable to chromosome aging. It is noteworthy that much of the mechanistic data available from human oocytes come from oocytes collected after hormonal stimulation, which may affect oocyte quality [32], and additional studies using oocytes from non-stimulated treatment cycles are warranted. Importantly, however, a detrimental impact of increasing maternal age upon spindle integrity has been noted in metaphase-II human oocytes both from natural-cycle-stimulated [16] and non-stimulated cycles [17], alluding that MT dysfunction may be a bona fide contributor to age-related oocyte aneuploidy.

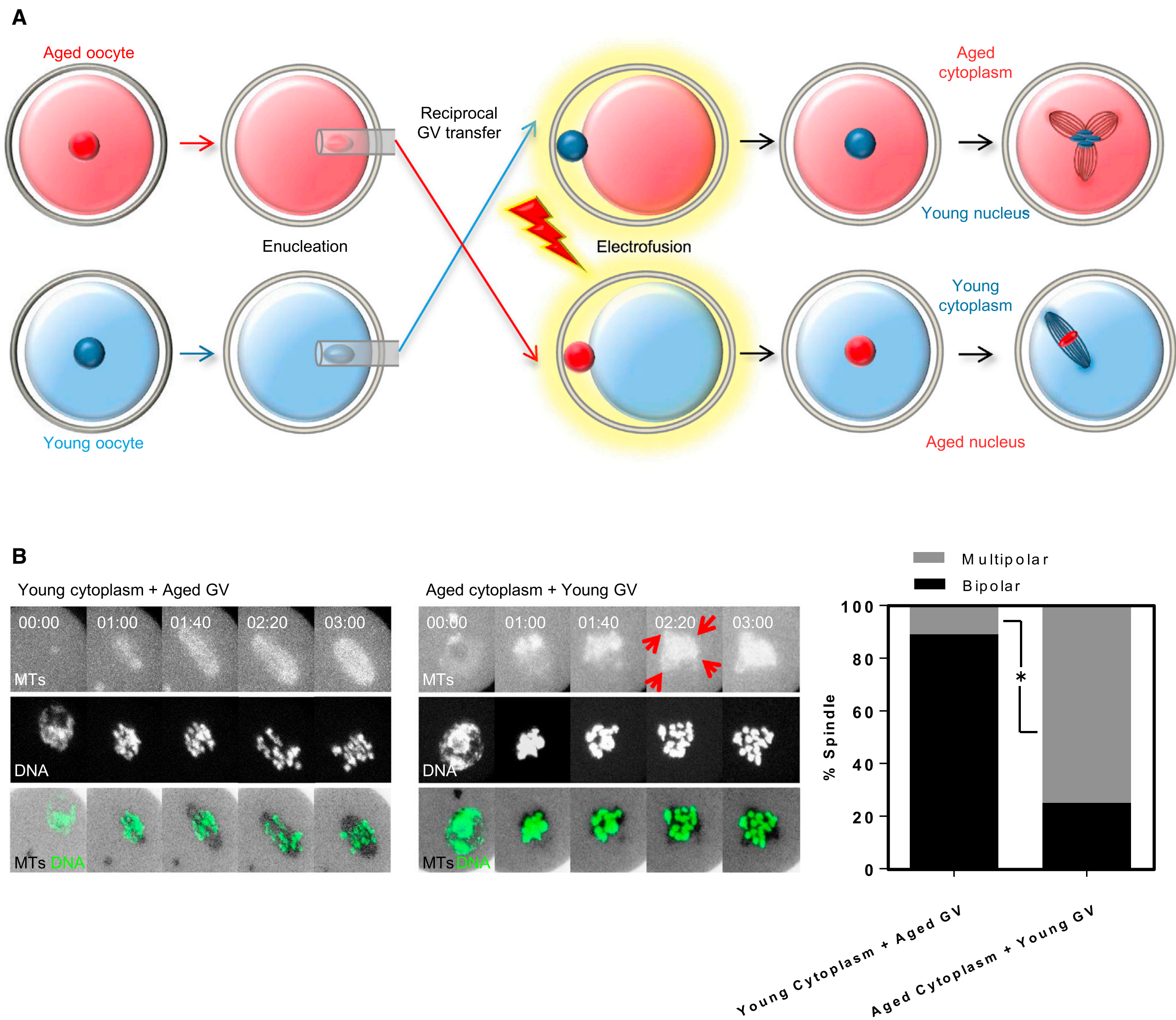

Figure 4. Transient Multipolar Spindle Formation Is Driven by Aging of the Cytoplasm, Not the Nucleus

(A) Cartoon explaining the experimental strategy of reciprocal GV transfer between young and aged oocytes.

(B) The resulting chimeric oocytes were subjected to live-cell imaging. Quantification of transient multipolar spindle formation in GV-transferred oocytes is shown to the right. Only one oocyte showed multipolar spindle formation when young cytoplasm fused with aged GV (11%; 1/9). In contrast, multipolar spindles (red arrows) were common in aged cytoplasm fused with young GVs (75%; 6/8). Asterisk indicates CHI2 $p < 0.01$. Meiosis-I completion rate (polar body extrusion) was not different between groups (aged-GV/young cytoplasm 78%; young GV/aged cytoplasm 75%).

See also Figure S4.

The molecular details underpinning faulty MT dynamics remain to be determined. One appealing notion is that potential mitochondria dysfunction in aged oocytes might reduce spindle ATP supply [33], especially because the spindle and mitochondria exist in close apposition in oocyte meiosis-I [34]. Alternatively, we speculate that altered expression or function of MT-organizing proteins and motors [8] may disrupt normal spindle assembly, perhaps increasing MT attachment errors without increasing the capacity to correct them. Whether experimental re-establishment of cytoskeletal stoichiometry might lessen errors in the highly error-prone environment of the aged oocyte remains to be seen.

EXPERIMENTAL PROCEDURES

Oocyte Collection and Handling

Germinal vesicle (GV) oocytes were collected from 3-month-old (young oocytes) and 12- to 15-month-old (aged oocytes) CD1 mice (Harlan and Charles River Laboratories). Young and aged oocytes were collected 44–46 hr post pregnant mare's serum gonadotrophin (young 5 IU; aged 10 IU) injection. All animal experiments were approved by the Comité Institutionnel de Protection des Animaux du CHUM (CIPA). Oocytes handling and four-dimensional (4D) live imaging were performed in M2 and M16 medium (Sigma) at 37°C for EB1:EGFP comets assay and long-term live imaging, respectively. Where necessary, GV oocytes were incubated with 200 μM 3-isobutyl-1-methylxanthine (IBMX) (Sigma) to maintain at GV stage.

Manufacture and Microinjection of mRNA

H2B:RFP and EB1:EGFP were microinjected into GV oocytes to visualize chromosomes and microtubules, respectively. Plasmid DNAs were amplified and linearized by appropriate restriction enzymes, and in vitro transcription was performed using mMessage mMachine (Ambion) according to the manufacturer's instructions, as previously [35]. mRNA was microinjected using Narishige micromanipulators mounted on a Leica inverted microscope [36].

Enucleation and Nuclear Transfer

Enucleation was performed upon IBMX-arrested oocytes 3 hr after mRNA microinjection. GV oocytes were incubated with M2 medium supplemented with 5 μg/mL cytochalasin B (Sigma) and 10 μM nocodazole (Sigma) for 10 min at room temperature. The zona pellucida was cut with a glass microneedle to allow insertion of an enucleation pipette (outer diameter ~30 μm). GV transfer was carried out as previously reported with some modifications [37]. Briefly, the GV was inserted between enucleated cytoplasm and zona pellucida and then transferred into 300 mM mannitol solution for electrofusion. Electrofusion was induced by a single pulse of direct current, 180–200 V/mm, for 99 μs. Fusion was confirmed 1 hr after electrofusion procedure.

Imaging and Data Analysis

Oocytes were fixed using 4% paraformaldehyde for 30 min and permeabilized using 0.25% Triton-X for 10 min [38]. The following primary antibodies were used in this study: α-tubulin (Sigma; 1:1,000); CREST (gift from William Earnshaw; 1:200); and pericentrin (Sigma; 1:500). Alexa-labeled secondary antibodies were purchased from Life Technologies. All imaging was performed on a Leica SP8 confocal microscope. For live imaging, a 20× 0.8 numerical aperture (NA) objective and HyD detector was used with laser illumination at a maximum of 0.2% laser power. Meiosis-I completion rates (polar body extrusion) were ~80% in all experiments both for young and aged oocytes. For rapid acquisition imaging of EB1:GFP comets, images were obtained at 2.578-s intervals and kymographs assembled using Fiji. Data were analyzed using Fiji and GraphPad Prism 7 software. Where shown, error bars indicate SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2017.02.025.

AUTHOR CONTRIBUTIONS

G.F. initiated the study. S.N. performed all experiments. S.N. and G.F. analyzed data and wrote the manuscript.

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