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Chromosome cohesion decreases in human eggs with advanced maternal age

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Summary

Aneuploidy in human eggs increases with maternal age and can result in infertility, miscarriages, and birth defects. The molecular mechanisms leading to aneuploidy, however, are largely unknown especially in the human where eggs are exceedingly rare and precious. We obtained human eggs from subjects ranging from 16.4 to 49.7 years old following *in vitro* maturation of oocyte-cumulus-complexes (OCCs) isolated directly from surgically-removed ovarian tissue. Whereas maternal age negatively impacted the total number of OCCs collected per subject, it did not appear to impact the ability of the gamete to resume meiosis and form a mature egg. We used a subset of these eggs to investigate how age-associated aneuploidy occurs in the human. The inter-kinetochore distance between sister chromatids increased significantly with maternal age, indicating weakened cohesion. Moreover, we observed unpaired sister chromatids from females of advanced age. We conclude that loss of cohesion with increasing maternal age likely contributes to the well-documented increased incidence of aneuploidy.

Keywords

maternal age; meiosis; cohesion; human; egg; aneuploidy

Fertility decreases with advanced maternal age (Van Voorhis, 2007). This decrease is primarily due to poor egg quality, as successful pregnancies increase significantly in females of advanced maternal age when eggs from young, fertile donors are used (Van Voorhis, 2007). An aneuploid egg, i.e., one with an incorrect chromosome complement, is known to be of poor quality. In humans, egg aneuploidy is associated with advanced maternal age, and most chromosome segregation errors occur during meiosis I (Hassold & Hunt, 2001).

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Meiosis in females is particularly error prone, likely a consequence of being protracted and involving two cell cycle arrests. In humans, oocytes enter meiosis during fetal development and arrest in prophase of meiosis I (prophase I). This prophase arrest is maintained until ovulation when, in response to hormonal cues, meiotic resumption occurs. Homologous chromosomes are segregated with completion of meiosis I, and the oocyte proceeds directly into meiosis II, arresting at metaphase of meiosis II (MII) at which point it is called an egg. If the egg is fertilized, meiosis II is completed with separation of sister chromatids. Because the physiologically-relevant follicle pool is thought to be fixed and non-renewable, a primordial follicle activated to grow in a female of advanced maternal age contains an oocyte that has been arrested in prophase I for decades.

Several molecular mechanisms have been proposed to explain the meiotic origins of aneuploidy, including errors in recombination, improper spindle formation and microtubule-kinetochore interactions, and defects in the spindle assembly checkpoint (reviewed in Hunt and Hassold, 2008; Hassold and Hunt, 2009; Eichenlaub-Ritter *et al.*, 2010; Jones and Lane, 2012). Recent findings in mouse suggest that deteriorating chromosome cohesion that occurs during the extended prophase I arrest is also likely a significant cause of age-associated aneuploidy (reviewed in Chiang *et al.*, 2012; Jesserberger, 2012; Jones and Lane, 2012). Chromosome cohesion, mediated by a multi-protein cohesin complex, is established along the chromosome arms and at the centromere, and serves to keep homologous chromosomes and sister chromatids together until completion of meiosis I and II, respectively (Watanabe, 2005; Holt and Jones, 2009). Current evidence suggests that in the oocyte cohesins load during S phase prior to recombination during fetal development and that little, if any, turnover occurs after this time (Revenkova *et al.*, 2010; Tachibana-Konwalksi *et al.*, 2010). Thus, chromosome cohesion must remain functional for months in the mouse and years in the human to ensure faithful chromosome segregation during meiosis. In several mouse strains, cohesion function is compromised in eggs from animals of advanced maternal age (Chiang *et al.*, 2010; Lister *et al.*, 2010; Chiang *et al.*, 2011; Merriman *et al.*, 2012). Compared to eggs from younger mice, those from older mice have reduced levels of chromosome-associated REC8, a meiotic-specific cohesin, resulting in increased inter-kinetochore distances (Chiang *et al.*, 2010; Lister *et al.*, 2010; Merriman *et al.*, 2012). These changes in cohesion precede and predict the most commonly observed chromosome segregation errors (Chiang *et al.*, 2010). These findings are also consistent with functional gene deletion studies in which loss of the cohesin component, SMC1 β , results in aneuploidy that is exacerbated with age (Revenkova *et al.*, 2004; Hodges *et al.*, 2005).

Although cohesin proteins are conserved between humans and mouse, we do not know if a functional deterioration of these components is associated with advanced maternal age in the human (Garcia-Cruz *et al.*, 2010). Such studies have been hampered largely by the difficulty in obtaining mature gametes from reproductively young and older women. Here we collected oocyte-cumulus-complexes (OCCs) from 18 subjects who had their ovaries removed for medical indications (Fig. 1A). Most subjects had cancer and were undergoing ovarian tissue cryopreservation for fertility preservation (55% gynecologic, 11% breast, 17% pediatric, 6% hematologic, 11% other). These subjects ranged in age from 16.4 to 49.7 years (average: 33.6 ± 11.0 years) and had not received hormonal stimulation prior to surgery. A total of 166 OCCs were collected with an average of 9.2 ± 12.2 OCCs per subject (Fig. 1B; range: 0 to 48 OCCs per subject). These OCCs had varying layers of somatic cells surrounding the oocyte and likely originated from follicles less than 5 mm in diameter, as no large antral follicles were visible on the ovarian surface (Fig. 1A). We observed a maternal age effect, with the number of OCCs collected per subject decreasing with age (Fig. 1B). Only one OCC was collected from subjects who were forty years and older, of which there were five used in this study. In addition to the maternal age effect, fewer OCCs were

collected from subjects who had a history of previous cancer therapy compared to untreated subjects of similar age (Fig. 1B).

To determine if oocytes within these OCCs were meiotically-competent, *in vitro* maturation (IVM) in the presence of follicle stimulating hormone (FSH) and luteinizing hormone (LH) was performed using 112 OCCs from 10 subjects. Overall, $28.2 \pm 5.8\%$ of the oocytes reached MII (Fig. 1C). Interestingly, maternal age did not impact meiotic competence as $29.2\% \pm 7.5\%$ of oocytes within OCCs from subjects under the age of 30 years resumed meiosis and reached MII compared to $27.2\% \pm 9.6\%$ from subjects over the age of 30 years (Fig. 1D; $P > 0.05$). The meiotic-competence of the oocyte, however, was size-dependent as has been previously documented in several mammalian species (Miyara *et al.*, 2003; Tan *et al.*, 2009). Oocytes that resumed meiosis were significantly larger in diameter than those that did not. Oocytes that remained germinal vesicle (GV)-intact following IVM were $108.0 \mu\text{m} \pm 1.5 \mu\text{m}$ in diameter, whereas those that had undergone germinal vesicle breakdown (GVBD) or had reached MII were $115.4 \mu\text{m} \pm 1.1 \mu\text{m}$ ($P < 0.01$) and $112.8 \mu\text{m} \pm 0.9 \mu\text{m}$ ($P < 0.05$), respectively. The eggs derived from IVM had characteristic morphology with a small first polar body and a bipolar spindle asymmetrically positioned in the cortex (Fig. 1E). The chromosomes were condensed and tightly aligned on the metaphase plate, and the actin cytoskeleton was cortical with a slight thickening adjacent to the spindle (Fig. 1E). Human eggs derived by this method of IVM may have clinical implications in the field of fertility preservation as they could be fertilized or cryopreserved for a patient's future use (Fasano *et al.*, 2011). Thus, there is a pressing need to understand the quality of these eggs in order to determine their ultimate clinical utility.

We used 18 of the eggs obtained through IVM from a subset of six subjects of ages 16.4, 19.3, 22.5, 27.5, 33.1, and 37.3 years to assess how chromosome cohesion is affected by maternal age. As a readout of chromosome cohesion, we measured the distance between kinetochores of sister chromatids, or the inter-kinetochore distance, in eggs using an *in situ* chromosome spreading technique (Fig. 1F and Figs. 2A–F) (Duncan *et al.*, 2009). We found that the average inter-kinetochore distances increased gradually and significantly with subject age (Figs. 2A–F). The average inter-kinetochore distance increased from $0.82 \mu\text{m} \pm 0.03 \mu\text{m}$ in the youngest subject (16.4 years) to $1.1 \mu\text{m} \pm 0.03 \mu\text{m}$ in the oldest subject (37.3 years) (Fig. 2E; $P < 0.001$). In the human, this absolute increase of $0.28 \mu\text{m}$ in inter-kinetochore distance between age extremes is consistent with data in two mouse strains in which increases of $0.13 \mu\text{m}$ and $0.44 \mu\text{m}$ were reported (Chiang *et al.*, 2010; Merriman *et al.*, 2012).

In addition to measuring inter-kinetochore distances within individual eggs, we were also able to accurately count total kinetochores in $>85\%$ of the eggs examined (16/18) to assess chromosome status. Chromosome segregation errors were observed in 31% (5/16) of the eggs following IVM, and four of these eggs were from the two subjects over the age of 30 (Subject E and F; Fig. 2E–H). Subject F, who was 37.3 years old, had two eggs each with a set of unpaired sister chromatids indicative of a total loss of cohesion (F_1 and F_2 ; Figs. 2F–G). In addition to a set of unpaired sister chromatids, egg F_1 also had an improperly segregated chromosome pair that was separated from the egg chromosomes and was not associated with the polar body DNA (Fig. 2H). This pair resided within the egg because, without including this pair, egg F_1 would be hypoploid. Subject E, who was 33.1 years old, instead had two hyperploid eggs (E_3 and E_4 ; Fig. 2F). One egg had an extra chromosome pair (E_3) whereas the other had an extra unpaired sister chromatid (E_4) (data not shown). Subject B, who was 19.3 years old, had one egg (B_1) that had an extra unpaired sister chromatid (Fig. 2F and data not shown). The five eggs with chromosome segregation errors were not restricted to those with the largest average inter-kinetochore distance (B_1 , E_3 , E_4 , F_1 , and F_2 ; Fig. 2F).

Taken together, these results demonstrate for the first time that in human eggs there is a maternal-age associated deterioration of chromosome cohesion as indicated by increased inter-kinetochore distances between sister chromatids. In the oldest two subjects, this increased inter-kinetochore distance was also accompanied by chromosome segregation errors. Interestingly, the majority of the observed segregation errors involved the premature separation of sister chromatids which is a clear manifestation of a complete cohesion defect. Although the majority of the observed aneuploidies occurred in subjects over the age of 30, there was one instance of aneuploidy in a reproductively young subject. This finding suggests that factors in addition to age may result in an aneuploid egg, including occupational and environmental exposures as well as medical treatments. The eggs used for these studies were obtained from ovarian tissue, primarily from females with a cancer diagnosis, so future studies to investigate whether similar aging mechanisms are conserved in a non-cancer population are warranted. Moreover, understanding if and how cancer itself and various cancer treatments affect chromosome dynamics during meiotic progression will be important for the field of fertility preservation.

Deteriorating chromosome cohesion is a major contributing factor in the age-associated meiotic origins of aneuploidy in the human. If chromosome cohesion in humans is only established during S phase (which happens one time in the development of an oocyte) and little turnover occurs after oogenesis as has been reported in model organisms, it is unlikely that supplementation of exogenous cohesin components will rescue the deterioration (Watanabe *et al*, 2001; Revenkova *et al.*, 2010; Tachibana-Konwalski *et al.*, 2010). Instead, methods that prevent the deterioration of chromosome cohesion are more likely have a greater impact in reducing the incidence of aneuploidy. The findings reported here thus provide important new opportunities for intervention in age-related infertility and are consistent with the progressive aging of an established oocyte pool.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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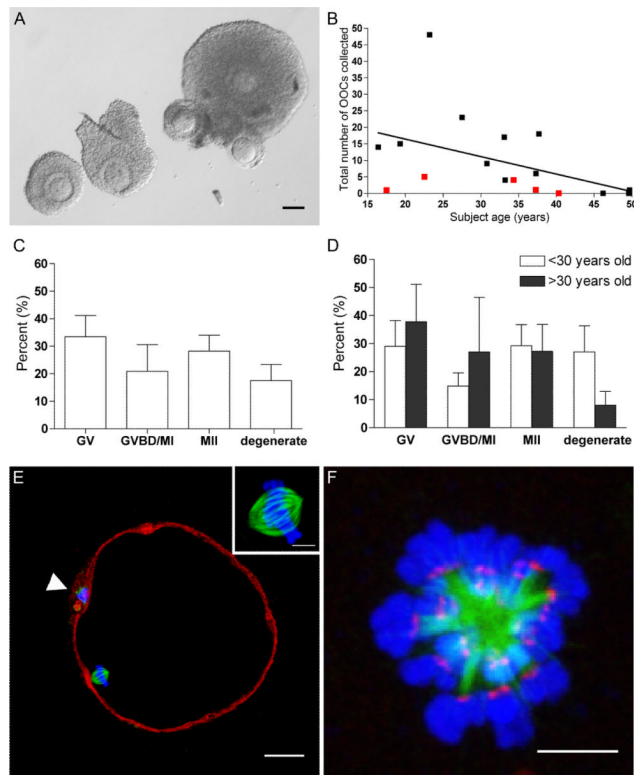


Figure 1.

Human MII eggs can be obtained following IVM of OCCs isolated directly from surgically-removed ovarian tissue. (A) Morphology of OCCs released from small antral follicles during ovarian tissue processing for cryopreservation. Scale bar = 100 μ m. (B) The effects of age and previous cancer therapy (red squares) on the number of OCCs collected per subject. (C–D) The percent of cells in each stage of meiotic progression following IVM for 36–42 hours (GV, germinal vesicle-intact; GVBD/MI, germinal vesicle breakdown/metaphase of meiosis I; MII, metaphase of meiosis II) reported as an (C) overall total or (D) separated according to age (> or < 30 years old). There were no statistical differences between the groups. (E) The cytoskeleton in a human MII egg following IVM. (F-actin = red; α -tubulin = green, DNA = blue). The polar body is marked by an arrowhead and the inset highlights the bipolar metaphase II spindle. Scale bar = 25 μ m. (F) The human meiotic spindle following monastrol treatment (α -tubulin = green, DNA = blue, kinetochores = red). Scale bar = 5 μ m.

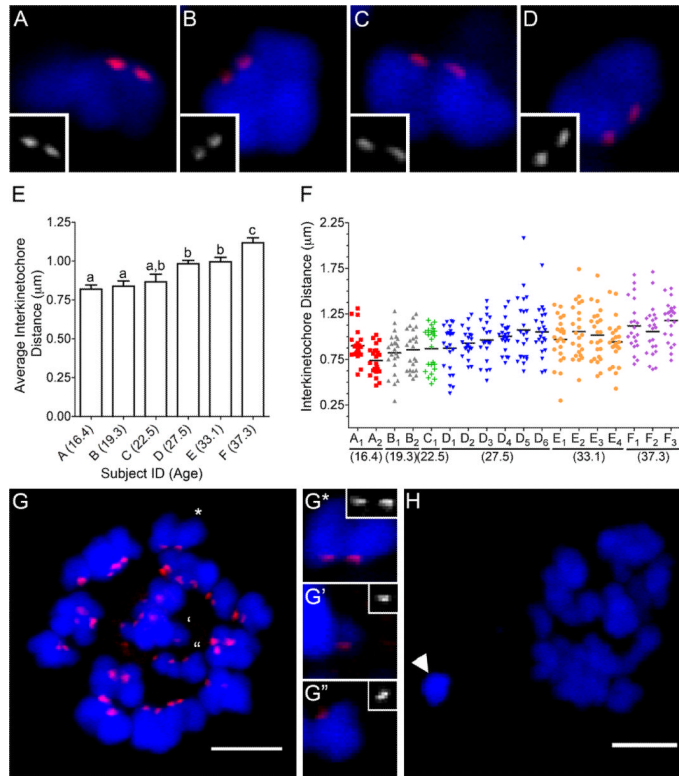


Figure 2. Inter-kinetochore distances increase significantly and chromosome segregation errors occur more frequently in human eggs with advanced age. Representative sister chromatids from (A–B) Subject D (27.5 years old) has on average smaller inter-kinetochore distances compared to those from (C–D) Subject F (37.3 years old). (E) The average inter-kinetochore distances of eggs from individual subjects are plotted with increasing maternal age. The uppercase letters correspond to the subject ID, and the subject age in years is indicated in parentheses. Different lowercase letters denote significant differences. (F) All the measured inter-kinetochore distances per individual egg are plotted with increasing maternal age. The uppercase letters, consistent with the labeling in (E), represent the subject ID and the subscript number indicates an individual egg. Each color represents inter-kinetochore measurements from the same subject. The mean inter-kinetochore distance for each egg is indicated by a black line. Eggs B₁, E₃, E₄, F₁, and F₂ were determined to have chromosome segregation errors. (G) An egg from Subject F (F₂) with a pair of separated sister chromatids. The marked chromosomes are further magnified to show a representative image of an intact sister chromatid pair (G*) and the pair of separated sister chromatids (G', G''); separated by four 0.2 μm-thick optical sections. (H) Another egg from Subject F (F₁) had a chromosome pair (arrowhead) that was separated from the egg DNA (**). Kinetochores = red and DNA = blue. The scale bar = 5 μm.