Inadequate histone deacetylation during oocyte meiosis causes aneuploidy and embryo death in mice

Tomohiko Akiyama, Masao Nagata, and Fugaku Aoki*

Department of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo, Chiba 277-8562, Japan

Edited by Barbara J. Meyer, University of California, Berkeley, CA, and approved March 10, 2006 (received for review December 20, 2005)

Errors in meiotic chromosome segregation are the leading cause of spontaneous abortions and birth defects. Almost all such aneuploidy derives from meiotic errors in females, with increasing maternal age representing a major risk factor. It was recently reported that histones are globally deacetylated in mammalian oocytes during meiosis but not mitosis. In the present study, inhibition of meiotic histone deacetylation was found to induce aneuploidy in fertilized mouse oocytes, which resulted in embryonic death in utero at an early stage of development. In addition, a histone remained acetylated in the oocytes of older (10-month-old) female mice, suggesting that the function for histone deacetylation is decreased in the oocytes of such mice. Thus, histone deacetylation may be involved in the fair distribution of chromosomes during meiotic division. The high incidence of aneuploidy in the embryos of older females may be due to inadequate meiotic histone deacetylation.

Mammalian female meiosis consists of specialized programs of cell division. Oocytes arrested at meiotic prophase I for long periods of time resume meiosis in response to hormonal stimulation and then proceed through meiosis I. After completion of this stage, during which the first polar body is extruded, oocytes successively enter meiosis II and are arrested again at metaphase II (MII). After fertilization, the second stage of meiosis is completed, with extrusion of the second polar body. Because, in this process, chromosomes are fairly distributed between the oocytes themselves and the polar bodies, there appears to be a meiosis-specific mechanism that functions in supporting a distinct pattern of chromosome distribution. Indeed, the oocytes of older females, in which many cellular functions have deteriorated (1), frequently fail in the fair distribution of chromosomes, which results in aneuploidy (2).

Although a mechanism supporting a fair distribution of chromosomes has not yet been identified, meiosis-specific changes in higher-order chromosome architecture may be involved in such a process. During extended meiotic prophase, chromosome morphology changes dynamically, allowing prophase to be divided into five stages (leptotene, zygotene, pachytene, diplotene, and diakinesis). Accurate chromosome segregation at meiosis I requires physical connections between homologous chromosomes to form hotspots for meiotic recombination (3). Chromatin at these regions displays a meiosis-specific increase in nucleosome sensitivity, suggesting that recombination events require alterations in chromatin structure (4). However, the mechanism responsible for a meiosis-specific chromatin configuration has not been clearly elucidated.

Posttranslational histone modifications are increasingly being recognized as playing important roles in chromosome structure and segregation during meiosis. Recently, a correlation between changes in histone modifications and chromosome structure during meiosis was reported in fungi and worms. Histones H3 and H4 in the vicinity of ade6-M26, a well-characterized site of meiotic recombination, were hyperacetylated during meiosis in fission yeast (5). In Caenorhabditis elegans, him-17 null mutants defective for meiotic recombination and chromosome segregation display a reduced and delayed accumulation of methylation on histone H3, Lys-9 (6).

In mammalian oocytes, the N-terminals of the nucleosome core histones, H3 and H4, are acetylated at prophase I, whereas they are deacetylated globally at MI and MII by histone deacetylase (HDAC) activity (7–9). However, the function of histone deacetylation during meiotic metaphase in oocytes is unknown. Although histone acetylation is involved in the regulation of gene expression (10–12), a transcriptionally inactive state has been already established in the oocytes at prophase I and is maintained during meiosis (13). Therefore, meiotic global histone deacetylation may instead be associated with changes in chromatin structure that regulate cellular functions other than gene expression.

In the present study, the role of histone deacetylation in the meiosis of mouse oocytes was analyzed. The results showed that inhibition of histone deacetylation during meiosis induces a high frequency of aneuploidy and embryo death. In addition, histone deacetylation was obtained from older mice frequently remained acetylated during meiosis, suggesting that a deficiency in the mechanism regulating histone deacetylation is associated with a high frequency of aneuploidy in oocytes from older females.

Results

Prophase-I-arrested mouse oocytes with germinal vesicles (GVs) spontaneously resume and complete meiosis I in vitro and thereafter arrest at MII stage. Resumption and completion of meiosis I is marked by GV breakdown and extrusion of the first polar body, respectively. We therefore first investigated whether inhibition of histone deacetylation affects this progression of meiotic maturation. Deacetylation was inhibited by culturing the oocytes in vitro with trichostatin A (TSA), a histone deacetylase-specific inhibitor (14). Inhibition of histone deacetylation was confirmed by immunostaining with an antibody (Ab) that reacts specifically with acetylated Lys-12 of histone H4 (H4K12) (Fig. 1). All of the oocytes treated with TSA underwent GV breakdown, and 66% emitted the first polar body (Table 1). These indices for the progression of meiosis differed little from those measured in oocytes cultured without TSA, demonstrating that inhibition of histone deacetylation did not influence meiotic maturation.

Next, we examined the effect of inhibiting meiotic histone deacetylation on preimplantation development. GV-stage oocytes that had been allowed to proceed with meiotic maturation and reached the MII stage in the presence of TSA were fertilized in vitro, and their development until blastocyst stage was followed (Table 2). TSA treatment continued for 3 h after insemination, by which time the second polar body was emitted, and meiosis was completed. As a control, the oocytes were allowed to undergo meiosis without TSA treatment.

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: GV, germinal vesicle; MII, metaphase II; TSA, trichostatin A.

*To whom correspondence should be addressed at: Department of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo, Kashiwa, Chiba 277-8562, Japan. E-mail: aoki@k.u-tokyo.ac.jp.

© 2006 by The National Academy of Sciences of the USA
embryos. Planted with control embryos the average was 1.8 resorbed females transplanted with TSA-treated embryos contained an transferred into recipients (Fig. 2 in utero as supporting information on the PNAS web site). Embryo death different from that of control pups (see Fig. 5, which is published the other live pups, and their rate of growth was not significantly weeks after birth. However, abnormalities were not observed in treated embryos, 1 died soon after birth, and 2 died by 2 or 3 weeks after birth. Among the 26 offspring observed in three females, and in two females, cesarean section was performed after noon on day 20. Among the 26 offspring obtained from the seven females transplanted with the TSA-treated embryos, 1 died soon after birth, and 2 died by 2 or 3 weeks after birth. However, abnormalities were not observed in the other live pups, and their rate of growth was not significantly different from that of control pups (see Fig. 5, which is published as supporting information on the PNAS web site). Embryo death in utero was investigated on day 11 by examining the embryos transferred into recipients (Fig. 2B). The result revealed that females transplanted with TSA-treated embryos contained an average of 5.3 resorbed embryos, whereas in females transplanted with control embryos the average was 1.8 resorbed embryos.

Table 1. Effect of TSA treatment on meiotic maturation

<table>
<thead>
<tr>
<th>Condition*</th>
<th>No. of oocytes cultured</th>
<th>No. (%) of oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GVBD</td>
</tr>
<tr>
<td>TSA−</td>
<td>159</td>
<td>159 (100)</td>
</tr>
<tr>
<td>TSA+</td>
<td>122</td>
<td>122 (100)</td>
</tr>
</tbody>
</table>

*The GV-stage oocytes were cultured for 18 h with TSA (TSA+) or without TSA (TSA−).

We postulated that the death of TSA-treated embryos after implantation was due to the generation of aneuploid oocytes resulting from chromosomal segregation errors. To address this possibility, karyotypic status was analyzed in one-cell zygotes that had completed oocyte meiosis (Fig. 3A). Hypo- and hyperploidy was observed in 32.7% and 24.5% of the TSA-treated one-cell zygotes (n = 49) compared with 8.3% and 10% of control zygotes, respectively (n = 60) (Fig. 3B). Furthermore, 34% of the TSA-treated oocytes that matured to the MII stage (n = 32) showed aberrant chromosome alignment: some chromosomes were scattered apart from the metaphase plate in the cytoplasm (Fig. 3C). There was no abnormality in the control oocytes treated without reagent (n = 34). Moreover, in most of the TSA-treated oocytes, chromosomal condensation appeared to be looser than that in the control oocytes. These results

Table 2. Effect of TSA treatment on preimplantation development

<table>
<thead>
<tr>
<th>Condition*</th>
<th>No. of embryos examined</th>
<th>No. (%) of embryos developed into</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2-Cell stage</td>
</tr>
<tr>
<td>TSA−</td>
<td>33</td>
<td>29 (87.9)</td>
</tr>
<tr>
<td>TSA+</td>
<td>50</td>
<td>46 (92.0)</td>
</tr>
</tbody>
</table>

*Oocytes were treated with TSA from the GV stage until 3 h after insemination (TSA+) or only immediately after insemination until 3 h later (TSA−).
demonstrate that the inhibition of histone deacetylation during oocyte meiosis induced aneuploidy, which led to embryo death.

In humans and mice, aneuploidy is predominantly caused by aberrant female meiotic chromosome segregation, and its frequency increases dramatically with maternal age (15–18). Therefore, the acetylation state of histone H4K12 during oocyte meiosis was studied in older (10-month-old) mice (Fig. 4). In 40% of MII-stage oocytes obtained from these mice (n = 81), histone H4K12 remained acetylated, whereas it was completely deacetylated in oocytes from young (3-week-old) mice (n = 74). We also examined the acetylation state of histones H3K14, H4K8, and H4K16. Histone H4K8 was distinctly acetylated in most of the MII-stage oocytes obtained from older mice, although only a faint level of acetylation was detected in almost all of the oocytes from young mice. When their signal intensities were quantified, a significant difference was detected (P < 0.005, Student’s t test; see Fig. 6, which is published as supporting information on the PNAS web site). Histones H3K14 and H4K16 were deacetylated in the oocytes from older mice as well as young mice. These experiments were performed by using superovulated oocytes. To exclude the possibility that the treatment for superovulation affected the acetylation state of H4K12, we repeated the same analysis for histone acetylation in the older mice but with naturally ovulated oocytes. The results were essentially the same as those using superovulated oocytes (see Fig. 7, which is published as supporting information on the PNAS web site). These results suggest that the mechanism-generating histone deacetylation was disrupted during meiosis in older females, thus inducing a high frequency of aneuploidy.

Discussion

This study demonstrates that inhibition of histone deacetylation during female meiosis induces aneuploidy and embryo death in mice and that a histone remained acetylated during meiosis in older female mice. These results suggest that the increase in aneuploidy of oocytes from older females is caused by a deficiency in the mechanism that regulates histone deacetylation during meiosis.

The analysis of fertilized oocytes showed that TSA treatment during meiotic maturation induced a significant increase in the frequency of chromosomal abnormalities (Fig. 3). Thus, histone deacetylation seems to play a key role in preventing aneuploidy during female meiosis. In somatic cells and mammalian male germ cells, a spindle assembly checkpoint monitors chromosome alignment and spindle integrity during cell division (19, 20). In the absence of proper chromosome alignment, the cell cycle is arrested or anaphase is delayed, allowing the cell to correct errors that might otherwise produce aneuploid progeny. However, this checkpoint is missing or less stringent in mammalian oocytes. Disturbances in the alignment of chromosomes during female meiosis caused by either chemical exposure (21) or mutations that disrupt folliculogenesis (22) do not induce meiotic arrest or a delay in anaphase onset. When the spindle checkpoint is absent, a fair distribution of chromosomes during female meiosis requires that there be a female meiosis-specific mechanism for aligning chromosomes correctly and preventing aneuploidy. We propose that histone deacetylation is one such mechanism. Although TSA treatment had no effect on the progression of cell division during meiosis, it induced a high frequency of unequal chromosome distribution during meiosis.
resulting in aneuploidy (Fig. 3). These observations support the hypothesis that female meiosis is regulated by mechanisms different from those of mitosis or male meiosis, in which chromosomal errors are corrected or aneuploidic cells are eliminated. Instead, in female meiosis, a fair distribution of chromosomes is ensured by the deacetylation of nucleosomal histones and alterations in chromatin structure.

We previously reported that TSA treatment had no influence on the progression of meiotic maturation (7). This result was confirmed in the present study by using a different mouse strain and different culture medium. Recently, De La Fuente et al. (23) reported that exposure to TSA during meiotic maturation induces abnormal chromosome alignment at the MII stage in mouse oocytes. This result is consistent with our finding that TSA treatment during female meiosis induces aneuploidy in first-cleavage embryos. However, De La Fuente et al. (23) also found that TSA treatment inhibited the progression of meiotic maturation in half of the oocytes examined. This discrepancy in the effect of TSA treatment on meiotic maturation remains to be elucidated.

TSA seems to inhibit deacetylation of histones specifically during female meiosis. To our knowledge, TSA inhibits deacetylation of two molecules as well as histones. First, it appears that TSA can increase the levels of acetylated p53 protein in somatic cells (24). Thus, changes in some proteins whose expressions are controlled by p53 might be involved in the chromosomal abnormality during meiosis. However, because there is no transcriptional activity during meiosis (13), it is unlikely that changes in acetylation of p53 would be associated with aneuploidy by TSA treatment in oocyte. Second, it was reported that TSA promotes the cellular acetylation of α-tubulin (25). However, immunocytochemistry with a specific Ab against acetylated α-tubulin showed that TSA did not affect acetylation level of α-tubulin during female meiosis (F.A., unpublished data). Therefore, it is unlikely that aneuploidy in one-cell zygotes was caused by the increased acetylation of p53 or α-tubulin with TSA treatment.

In the present study, we prepared the control oocytes for TSA treatment during meiosis by treating the oocytes with TSA at the time of insemination (3-h periods after insemination). This control was designed to exclude the effect of TSA on fertilization. In this control, aneuploidy was detected at the frequency of 18.3% (Fig. 3). Although this value is markedly lower than that in the oocytes that had been treated with TSA during meiosis, it was much higher than those that have been reported in some previous studies (~1.5%) (16, 26). This high incidence of aneuploidy in our controls may have been due to the effects of in vitro maturation and fertilization. In previous studies that reported a low frequency of aneuploidy, one-cell zygotes were obtained from mated females: thus, they underwent meiotic maturation and fertilization in vivo (16, 26). However, several studies have shown that when GV-stage oocytes are matured to the MII stage in vitro, aneuploidy is observed in 11–33% of them (27–29). Moreover, Fraser and Maudlin (16) reported an increased incidence of aneuploidy (4.3%) in one-cell zygotes fertilized in vitro compared with those fertilized in vivo (1.5%). Therefore, it is likely that in vitro maturation and fertilization increase the frequency of aneuploidy. Indeed, we examined the frequency of aneuploidy in one-cell zygotes that had been matured to the MII stage in vitro and fertilized in vitro without any TSA treatment and found that aneuploidy occurred in 15.9%. Culturing the oocytes in vitro may induce the reduction of histone-deacetylating activity, resulting in the increased frequency of aneuploidy.

Aneuploidy has been estimated to occur in 10–25% of fertilized human oocytes and 0.3% of all human newborns; thus, numerical chromosomal abnormalities are the leading cause of pregnancy loss, congenital defects, and mental retardation (2). Almost all instances of aneuploidy derive from female meiotic errors, and increasing maternal age represents a well-documented risk factor. An important finding in the present study was that histone acetylation persisted in the oocytes from older female mice, suggesting that decreased histone deacetylation contributes to maternal-age-related aneuploidy in mammals. It has been reported that the frequency of meiotic chromosome errors markedly increases with maternal age. In mice, a significantly higher incidence of aneuploidy was observed in fertilized oocytes from older animals than in those from young ones (7.5% vs. 3.3%) (16). In humans, the chance of generating chromosomally abnormal fetuses increases from 6.8% in women 35–39 years of age to ~50% in those 45 years of age (30). Moreover, the risk of having a baby with Down's syndrome, trisomy 18, or other chromosomal abnormalities increases with increasing maternal age. Based on the age-associated increase of spontaneous abortions and numerical meiotic division errors, it has been suggested that an age-related decline in oocyte quality is associated with a higher incidence of aneuploidy (31). However, neither a molecular mechanism explaining the high incidence of aneuploidy in older mothers nor the presence of another factor determining oocyte quality and the even distribution of chromosomes has been reported. We have shown that histone H4K12 remained acetylated in 40% of the MII-stage oocytes of older mice, whereas histone deacetylation was complete in the oocytes of young mice (Fig. 4). Moreover, a higher signal of acetylated H4K8 was detected in MII-stage oocytes from older mice than from young ones, although some other lysines (H3K14 and H4K16) were deacetylated in the oocytes from older mice (Fig. 6). These results suggested that the ability of deacetylating histones had decreased, but not disappeared, in the oocytes from older females. Furthermore, in young females, the maintenance of histone acetylation after TSA treatment led to >50% aneuploidy in fertilized oocytes. Thus, remaining acetylation during meiosis seems to increase the incidence of aneuploidy in the oocytes.

Fig. 4. Histone acetylation persists in MII-stage oocytes from older mice. (A) MII-stage oocytes obtained from young (3 weeks) and older (10 months) female mice were immunostained with Ab against acetylated H4K12 (AcH4K12). The Ab was localized with FITC-conjugated secondary Ab (green), and DNA was stained with DAPI (blue). (B) Histone acetylation was completely undetectable in all oocytes from young mice but was frequently observed in the oocytes from old mice.
from older mice. In these oocytes, however, the level of histone acetylation was lower at the MII stage than the GV-stage, indicating that histones were deacetylated to some extent during meiotic maturation. This finding may be one reason why the frequency of aneuploid embryos (<10% of the frequency reported by most studies) is much lower than that of the embryos with acetylated histone H4K12 (40%) in older female mice. Taking these results together, we propose a mechanism in which histone deacetylation during meiosis is frequently disrupted in the oocytes of older females, resulting in a significant increase in the incidence of aneuploidy in their offspring.

Materials and Methods

Collection and Culture of Oocytes. Fully grown oocytes arrested at prophase I of meiosis were obtained by superovulating 3-week-old BDF1 female mice (CLEA Japan, Tokyo) by using 5 units of pregnant mare's serum gonadotrophin (PMSG). The ovaries were removed from the mice at 47 h after PMSG treatment and transferred into Hepes-buffered Whitten's medium (32) supplemented with 3 mg/ml BSA (Sigma). The ovarian follicles were punctured with 27-gauge needles to release oocytes enclosed with cumulus cells. The cumulus cells surrounding the oocytes were removed by gentle pipetting through a narrow-bore glass pipette, and the oocytes with a GV were collected. The oocytes then were transferred into fresh Whitten's medium and incubated in a humidified atmosphere of 5% CO₂/95% air at 38°C. For oocytes fertilized in vitro after the progression of meiosis, GV-stage oocytes were cultured in Waymouth's medium instead of Whitten's medium as described in ref. 33.

In Vitro Fertilization and Embryo Cultures. Sperm were collected from the caudal epididymis of adult ICR male mice (SLC Japan, Shizuoka, Japan) and capacitated by preincubation for 1 h in human tubal fluid (HTF) medium (34) supplemented with 30 mg/ml BSA. MII stage oocytes, cultured in HTF medium for 18 h, were inseminated with capacitated sperm in a humidified atmosphere of 5% CO₂/95% air at 38°C. Three hours after insemination, the fertilized oocytes were washed and cultured in K-modified simplex optimized medium (KSOM) (35) containing 3 mg/ml BSA.

TSA Treatment. Histone deacetylase activity during meiosis was inhibited by treating GV-stage oocytes with 100 nM TSA (Sigma) added to the culture medium for 18 h. A stock solution of TSA (1 mM) dissolved in water was diluted with culture medium immediately before use. Oocytes fertilized after meiosis progression in vitro were inseminated with sperm in the presence of TSA and cultured for 3 h, during which time the second polar body was emitted, and meiosis was completed. The fertilized oocytes then were washed with K⁺-modified simplex optimized medium and cultured. The presence of TSA did not affect the rate of fertilization. As a control, GV-stage oocytes were cultured in vitro in the absence of TSA, and then oocytes that had reached MII stage were treated with TSA from the time immediately after insemination until 3 h later.

Embryo Transfer. Embryos that had reached the two-cell stage were transferred into the oviducts (7–11 embryos per oviduct) of ICR females (SLC Japan) mated during the previous night with vasectomized ICR males. On day 19 (day 0 was the day when the vaginal plug was observed), the number of pups born was recorded, and live pups were nursed by lactating ICR females. Recipient females that did not give birth naturally until noon on day 20 were killed; the pups were quickly removed from the uteruses, and live pups were raised by lactating females. The body weights of the pups were measured weekly. The number of embryos lost was assessed by killing the recipients on day 11 and then recording the number of implantation sites and fetuses.

Chromosomal Analysis of One-Cell Zygotes. At 11 h after insemination, one-cell zygotes were incubated in K⁺-modified simplex optimized medium containing 0.1 μg/ml colcemid to arrest the cells at the first-cleavage metaphase. Cytogenetic preparation of the zygotes was performed according to procedures described in ref. 36 with some modifications. In brief, 6 h after colcemid treatment, zygotes were transferred into a hypotonic solution of 0.9% sodium citrate for 10 min at room temperature and then exposed to a freshly prepared fixative mixture of 3:1 methanol:acetic acid. Chromosomes were air-dried and stained with Giemsa (Sigma), and the number of chromosomes was scored by two independent observers.

Collection of Oocytes from Older Females. MII-arrested oocytes from older mice were obtained by superovulating 10-month-old ICR female mice (Charles River Japan, Kanagawa, Japan) with 5 units of pregnant mare's serum gonadotrophin (PMSG), followed 48 h later with 5 units of human chorionic gonadotropin (hCG). Cumulus–oocyte complexes were collected from the ampullae of oviducts 15 h after hCG injection, placed in Whitten's medium, and freed of cumulus cells with hyaluronidase. As a control, oocytes from 3-week-old ICR female mice (Charles River Japan) were used. These younger mice had been subjected to the same hormonal treatment as the older ones.

In some experiments, naturally ovulated oocytes instead of superovulated ones were used. The vaginal smears of the females were examined daily to check their estrous cycles, and naturally ovulated oocytes were obtained during the next morning of the proestrus stage. In these experiments, 5- to 7-week-old mice were used as controls instead of 3-week-old mice.

Immunocytochemistry. The oocytes were fixed with 3.7% paraformaldehyde overnight, permeabilized with 0.5% Triton X-100 for 15 min, and then immunostained with an Ab against acetylated H4K12, H3K14, H4K8, or H4K16 (all from Upstate Biotechnology, Lake Placid, NY) for 1 h at room temperature. Ab that bound to the oocytes was probed with FITC-conjugated anti-rabbit IgG (Jackson ImmunoResearch). To visualize microtubules, some oocytes were double-stained with antiacetylated H4K12 and anti-α-tubulin Ab (Sigma), which were probed with rhodamine-conjugated anti-mouse IgG (Chemicon). DNA was visualized by counterstaining the oocytes with diamidino-2-phenylindole (DAPI). Fluorescence was detected by using the Leica spectral confocal scanning system.

We thank H. Miki and A. Ogura (RIKEN Bioresource Center) for helpful comments on the chromosomal analysis and T. Hata, O. Suzuki, and J. Matsuda (National Institute of Biomedical Innovation) for technical advice on embryo transfer. This work was supported in part by Ministry of Education, Science, and Culture Grants HD 1430614 and 16045203 (to F.A.).