Chromatin acetylation in human oocytes

Acetylacja chromatyny w ludzkich oocytach

Alena Langerova

GENNET, Prague, Czech Republic,

Abstract

Objectives: *The frequency of aneuploidies in human oocytes is extremely high. It is hypothesized that the cause may be due to abnormal chromatin (histone) acetylation/deacetylation. The aim of our study was to analyzed the acetylation/deacetylation pattern in spare human oocytes.*

Materials and Methods: *Human spare oocytes (311), in other words oocytes that were not mature when collected from follicles or control oocytes (bovine, mouse), were fixed with paraformaldehyde and then labeled with antibodies against acetylated histones.*

Results: *Labeling against AcH4/K12 or hyperacetylated H4 showed high intensity of the fluorescence signal in all immature (germinal vesicle staged) and approximately 50% of the maturing (mature) oocytes.*

Conclusion: *In conclusion, the labeling of human oocytes (chromosomes) showed very inconsistent patterns of acetylation/deacetylation, what may suggest they did not reach the metaphase II stage at the time of follicle aspiration, and were epigenetically abnormal. It may also explain the high frequency of chromosomal abnormalities in human oocytes.*

Key words: **oocytes** / **maturation** / **chromosomes** / **acetylation** / **aneuploidies** /

Streszczenie

Cel: *Częstość aneuploidii w ludzkich oocytach jest niezmiernie wysoka. Istnieją hipotezy, że jest to związane z nieprawidłową acetylacją/deacetylacją chromatyny (histonów). Celem badania była ocena wzoru acetylacji/deacetylacji w niewykorzystanych ludzkich oocytach.*

Materiał i metoda: *Ludzkie niewykorzystane oocyty (311), innymi słowy oocyty niedojrzałe w momencie pobrania z pęcherzyków lub oocyty kontrolne (krowie, mysie), zatopiono w paraformaldehydzie i oznakowano przeciwciałami przeciwko acetylowanym histonom.*

Wyniki: *Znakowanie przeciwko AcH4/K12 lub hiperacetylacja H4 wykazały wysoką częstość sygnału fluorescencyjnego we wszystkich niedojrzałych (w stadium pęcherzyka germinalnego) i w około 50% dojrzałych oocytach.*

Corresponding address:
Alena Langerova
GENNET
Kostelni 9, CS-170 00 Prague 7, Czech Republic
Phone: + 420 776 816 257; Fax: + 420 242 456 722
e-mail: aja.langerova@seznam.cz

Otrzymano: **12.10.2012**
Zaakceptowano do druku: **15.03.2013**

Wnioski: *Znakowanie ludzkich oocytów (chromosomów) wykazuje wysoce niejednoznaczny model acetylacji/deacetylacji, co może sugerować, że nie osiągają one metafazy II podziału w momencie aspiracji pęcherzyka i są epigenetycznie nieprawidłowe. Ten fakt może tłumaczyć wysoką częstość nieprawidłowości chromosomalnych w ludzkich oocytach.*

Słowa kluczowe: **oocyt /dojrzewanie /chormosom /acetylacja /aneuploidia /**

Introduction

The frequency of aneuploidies in human oocytes is very high and up to 60% of them have been reported to be chromosomally abnormal [1]. The incidence in humans seems to be much higher than in other mammals: mouse – approximately 5%, cattle – 10%, pig – 10% [2]. It must be, however, emphasized that these estimations often differ, depending on the technique used. The reason why human oocytes are so often chromosomally abnormal remains undiscovered. In fact, the basis for aneuploidies can be established long before they can be detected in fully grown oocytes [3]. Hunt and Hassold [3] defined three developmental phases of oogenesis when the quality of oocytes can be influenced – i/ oogenesis, ii/ follicle formation and iii/ oocyte growth and maturation. The research in this field in humans is mostly oriented towards the final phase of the oocyte development: its maturation covering the period when the oocyte undergoes germinal vesicle breakdown and subsequently reaches the metaphase II stage [4]. Highly dynamic morphological changes and specific histone modifications were reported to occur in mammalian oocytes within that time period. An immature oocyte contains a prominent nucleus (germinal vesicle – GV), in which a well-visible nucleolus can be seen in some species (rodents, humans, pig). A germinal vesicle breakdown (GVBD), in other words the dissolution of GV membrane, disappearance of nucleoli and gradual condensation of chromatin, occurs following a gonadotropin surge or in in vitro cultures. The condensed chromosomes are then arranged in the metaphase I stage (MI) with a well-formed spindle. This stage is followed by a short anaphase to telophase I (A-TI) transition, during which a half of the oocyte chromosome complement is extruded from the oocyte in the form of the first polar body (PBI). The chromosomes are again arranged on a metaphase plate (metaphase II, MII).

In most mammals, the oocyte is released from the follicle at that stage and then fertilized by the sperm. The histone modifications studies are mostly focused on acetylation and methylation, while the most exhaustive experiments were performed in mice. Methylation of chromatin has been reported to be rather constant and positive labeling was detected not only on condensed chromosomes (MI, A-TI, MII), but also in the nuclei [5, 6]. Quite different situation was, however, observed when antibodies against acetylated histones were used. In general, germinal vesicles were consistently positively labeled but as soon as chromosomes condensed (MI, MII), no labeling was detected. The only exception is the anaphase to telophase I (A-TI) transition with a weak signal on chromosomes [7, 8, 9, 10]. It must be noted, however, that a different pattern of labeling was detected in pigs, where chromatin remained acetylated [11].

Interestingly, the oocyte chromosome acetylation has been shown to increase with age of the female and also some IVF (in vitro fertilization) techniques may be the influencing factor [12, 13, 14]. A clear association between the aberrant acetylation pattern and the unequal segregation of chromosomes has been demonstrated in mice [15, 16]. Whilst in oocytes isolated from young females no positive labeling was detected on condensed chromosomes, in oocytes from old females about half of the metaphases showed a positive signal. The increased acetylation of chromosomes was also detected in oocytes aged in culture (MII). Thus, it is logical to assume that similar situation may exist in humans, too. As mentioned above, human oocytes are often chromosomally abnormal and thus one can expect that the analysis of chromosome acetylation pattern will correlate with aneuploidies. To the best of our knowledge, only two papers on the acetylation in human oocytes were published after the completion of our study [17, 18]. We analyzed the pattern of acetylation in spare human oocytes, i.e. those that did not reach the metaphase II stage at the time of their aspiration from the follicles or eventually remained unfertilized after IVF.

Material and methods

Human oocytes, after standard stimulation, were collected by aspiration from follicles and immediately morphologically evaluated. Written consent was obtained from all couples and the study was approved by the Ethical Board of GENNET. In total, we analyzed 311 oocytes obtained from 152 patients, aged from 26 to 38 years, treated at GENNET for a wide range of infertility problems (endometriosis, tubal factors, PCOS - polycystic ovarian syndrome, anovulation, genetic factors, male factors, etc.). The mature oocytes, i.e. the metaphase II staged, were used either for ICSI or were mixed with spermatozoa. The remaining oocytes were briefly cultured (2-3h) in Quinns Advantage Protein Plus Cleavage Medium (SAGE IVF, Trumbull, CT, USA) at 37°C and 5% CO_2, during that period some oocytes expelled first polar bodies. Thereafter, zonae pellucidae were dissolved by pronase (0.5%) in PBS (phosphate buffered saline) and then the oocytes were fixed with 4% paraformaldehyde (PFA) in PBS for 30min. The oocytes were then kept in PBS at 4°C until immunolabeling. Before labeling the oocytes were permeabilized by Triton X-100 (0.2% in PBS) for 10min and then blocked overnight in PBS supplemented with 1% BSA (bovine serum albumin) and 0.1% TritonX-100 at 4°C. The oocytes were then incubated overnight with one of the following antibodies: anti-acetylated H4/K12 (1:800, Upstate) and anti-hyperacetylated H4 (1:1000, Upstate). These antibodies were extensively characterized in our laboratory [5] or were most widely used in studies analyzing the acetylation/deacetylation processes during oocyte maturation in different mammals (anti-acetylated H4/K12). Following the incubation with one of the above mentioned antibodies, the oocytes were washed several times in PBS/BSA and then incubated for 2h

with the secondary antibody (1:400, donkey-anti rabbit, Jackson Immunoresearch). The oocytes were then washed several times in PBS/BSA, mounted on slides in Vectashield mounting medium with DAPI (Vector Laboratories) and examined under Olympus IX 71 fluorescence microscope. Each labeling was performed several times. Unless otherwise stated, all chemicals were purchased from Sigma.

Control animal oocytes

In order to exclude some technical problems in labeling, we used bovine and mouse oocytes at different stages of maturation as controls. Bovine oocytes were aspirated from large antral follicles and either fixed immediately (GV stage) or cultured in M199 supplemented with FCS (fetal calf serum, 10%), gentamicin (50µg/ml), Na-pyruvate (0.2 mM) and Pergonal (0.1 IU/ml, Serono, UK) for 18-20h at 38.5°C/5% $C0_2$ in the air (maturing – MI, mature – MII) and then fixed. Mouse oocytes were isolated from ovaries of PMSG (pregnant mare serum gonadotropin, 5 I.U.) stimulated mice (BDF1) and either fixed immediately (GV) or matured in MEM supplemented with BSA (4mg/ml), gentamicin (50 µg/ml) and Na-pyruvate (0.2 mM) at 37°/ 5% $C0_2$ in the air for up to 12h and then fixed. The oocytes of both species were treated and labeled similarly as described above for the human oocytes. As the animal oocytes can be easily distinguished from the human oocytes, they were often incubated in the same well.

Results

The results are summarized in Table I. It must be noted that we analyzed only the oocytes with no signs of degeneration, i.e. round-shaped with homogeneous cytoplasm.

First, we tested the antibody that was specific for AcH4/K12. Controls, i.e. bovine (25) and mouse (42) GV staged oocytes were always positively labeled, whereas in human oocytes 90% (95/105) of the immature oocytes showed positive labeling (Fig 1 a,b). No labeling at all was detected in the remaining ones. 47% of maturing MI stage oocytes were positively labeled (27/58), all telophase I (TI) were positive (Figure 2 a,b), and 49% (22/45) of the metaphase II oocytes showed a positive signal. Bovine and mouse maturing and mature oocytes showed mostly no labeling (90%).

Anti-hyperacetylated H4 was used as the second antibody. In that case, almost all human immature GV staged oocytes were positively labeled (95%; 35/37). About half of the metaphase I (48%; 40/83) and the metaphase II (50%; 9/18) was positive when this antibody was used, whilst the other half was without labeling. All telophase I stage oocytes were positively labeled.

The expected pattern of labeling was detected in controls, i.e. in bovine and murine oocytes. Here, all GV–staged oocytes were positively labeled (bovine – 15, murine – 25), in maturing oocytes no labeling was detected in almost all oocytes (45 – bovine, 56 – murine). Surprisingly, we found evident differences in labeling when comparing the pattern of labeling (AcH4/K12, anti-hyperacetylated H4) between oocytes obtained from the younger and the older patients, (under and over the age of 30, respectively). Similarly, we were unable to find any relationship between the pattern of labeling and a given fertility problem.

In conclusion, when compared to other species tested so far, our results demonstrate that the analysis of acetylation/deacetylation of chromatin in human oocytes gave us rather inconsistent results . At present, it would be very difficult to use this labeling to evaluate the quality of oocytes and to explain the high frequency of aneuploidies in them.

Discussion

The ovary contains basically three categories of oocytes: growing, intermediate and full-grown. Only full-grown oocytes can undergo germinal vesicle breakdown and reach the metaphase II stage [4]. This, however, does not mean that they are chromosomally normal and developmentally fully competent. For example, it has been convincingly demonstrated that those oocytes where the nucleolus is surrounded with a ring of chromatin are the best ones.

In general, GV (immature) oocytes of all mammalian species labeled so far with antibodies against acetylated histones were positive [19, 20, 21], but the use of these antibodies (anti-AcH4/K12, anti-hyperacetylated H4) also indicates that the extrapolation of the results obtained in laboratory and domestic animals to humans, in order to explain the frequency of aneuploidies, may be risky. Essentially, our results are in agreement with the observations of van der Berg et al. [18], who found that all immature oocytes (GV) labeled with anti-AcH4/K12 antibody and approximately 50% of MI and MII oocytes were positive. Moreover, detailed analyses demonstrate a positive correlation between positive labeling and abnormal spindle morphology. This may consequently lead to unequal distribution of the chromosomal material, either during the exit from the metaphase I or the metaphase II. Their results, however, also show that it will be difficult to find any relationship between the acetylation pattern and aberrant chromosome segregation. AcH4K12 seems to be the most convenient of all antibodies they tested (AcH4K5, AcH4K8, AcH4K12, AcH4K16). These authors also found certain differences in labeling (AcH4/K12) between the oocytes

Table I. Chromosone Acetylation in Human Oocytes.

Antibody	Oocyte maturation stage – No of oocytes (±; positive/negative labeling) [%]			
	GV	MI	A-TI	MII
AcH4K12	105 (95/10) [95/10]	58 (27/31) [47/53]	2 (2/0) [100/0]	45 (22/23) [49/51]
HyperAcH4	37 (35/2) [95/5]	83 (40/43) [48/52]	3 (3/0) [100/0]	18 (9/9) [50/50]

Abbreviations: GV – germinal vesicle, MI(II) - metaphase I(II), A/TI – anaphase/telophase I, GV category involves both SN (surrounded nucleolus, approximately 60%) and NSN (non-surrounded nucleolus) oocytes. As no differences between these two groups were detected, the data were pooled.

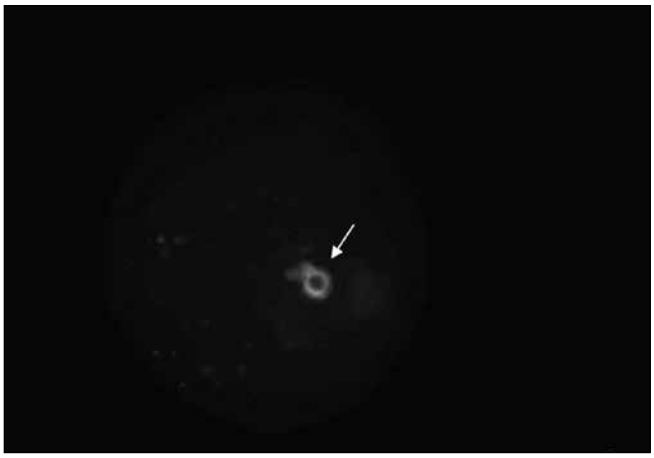

Figure 1A.) Immature germinal vesicle stage human oocyte with the nucleolus surrounded by chromatin (arrow) labeled with anti-AcH4/K12 antibody. All oocytes showed the positive chromatin labeling. The same situation was observed after labeling with anti-hyperacetylated H4 antibody. x 400.

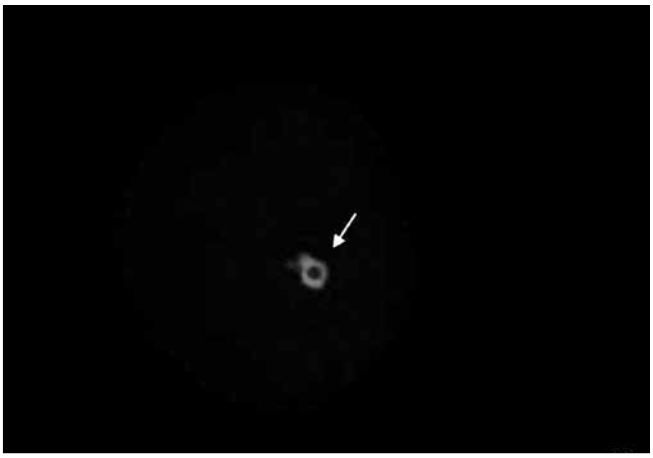

Figure 1B. Parallel DAPI staining.

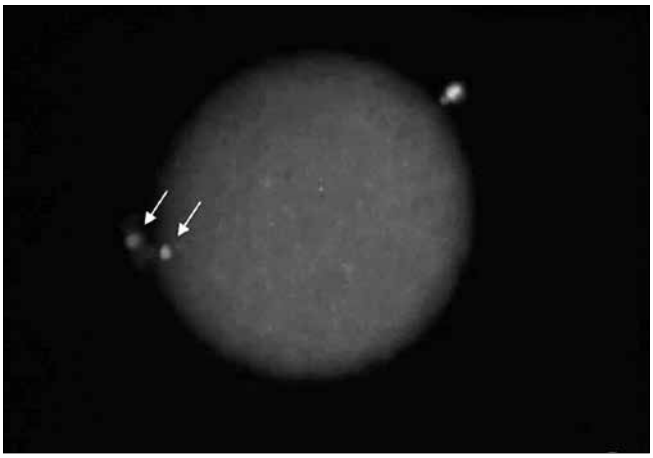

Figure 2A. Telophase I stage human oocyte with a weak chromosome groups (arrows) labeling (AcH4/K12). x 400.

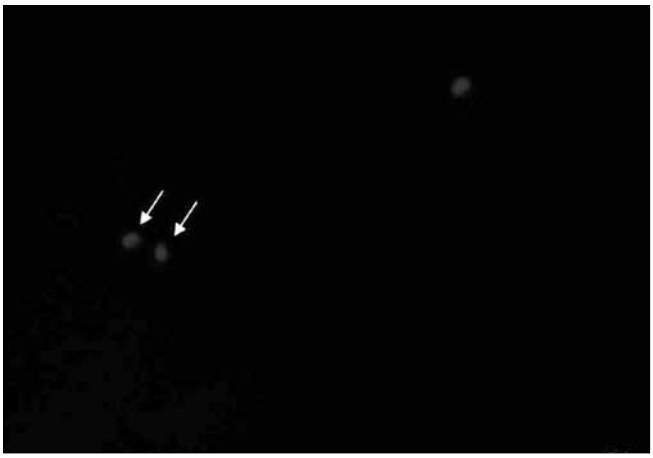

Figure 2B. Parallel DAPI staining.

from the young vs. the older patients. The first group contained fewer oocytes that were positively labeled with that antibody. We did not find these differences in our study. That, however, may be explained by different media that had been used for oocyte culture before they were fixed [22]. Slightly different results were reported by Huang et al. [17], who noted positive labeling for all GV-staged oocytes (AcH4K12, AcH3K9).

For AcH3K9 the percentage of positively labeled maturing and mature oocytes was below 50% in in vitro matured oocytes and almost all in vivo matured ones were without labeling. Essentially, the same situation was observed when AcH4K12 antibody was used. The explanation for these differences is very difficult and one cannot exclude that certain technical aspects, i.e. the oocyte culture, handling and labeling, might have influenced the overall pattern of labeling.

In general, the interpretation of our results poses considerable challenges. First, it must be noted that we used oocytes from patients with different fertility problems. Second, we analyzed only those oocytes that did not reach the MII stage at the time of their collection from follicles. The question remains whether these oocytes are somewhat abnormal when compared to more rapidly maturing oocytes. It has been clearly demonstrated that oocytes maturing faster are, after fertilization, developmentally more competent than those oocytes in which maturation lasts longer [23]. It has been also shown that certain manipulations and culture conditions influence the level of methylation (global DNA methylation and/or histone acetylation) in embryos [20, 24, 14]. Essentially, the labeling intensity in manipulated embryos was significantly higher than in controls. Manosalva and Gonzales [25] demonstrated in mice that the consistency of labeling decreases in GV stage oocytes with an increasing female age.

We hypothesize that there may be two possible explanations: a) human oocytes are distinct when compared to other mammalian species tested so far, b) the prevailing population of human oocytes is abnormal.

It is fairy difficult to determine these issues as human oocytes are primarily used for test tube babies production and spare, high quality oocytes, are available only in exceptional situations.

Clearly, more detailed analysis in this field is necessary to account for the high frequency of aneuploidies in human oocytes and embryos, as well as to develop some ways of their prevention [26, 27].

Acknowledgements
This work was supported from MZE0002701404.

The author declares no conflict of interest.

References

1. Jones K. Meiosis in oocytes: predisposition to aneuploidy and its increased incidence with age. *Hum Reprod Update.* 2008, 14, 143-158.
2. Hornak M, Jeseta M, Musilova P, [et al.]. Frequency of aneuploidy related to age in porcine oocytes. *PLoS One.* 2011, 6, e18892.
3. Hunt P, Hassold T. Human female meiosis: what makes a good egg go bad? *Trends Genet.* 2008, 24, 86-93.
4. Fulka Jr J, First N, Moor R. Nuclear and cytoplasmic determinants involved in the regulation of mammalian oocyte maturation. *Mol Hum Reprod.* 1998, 4, 41-49.
5. Fulka H. Changes in global histone acetylation pattern in somatic cell nuclei after their transfer into oocytes at different stages of maturation. *Mol Reprod Dev.* 2008, 75, 556-564.
6. Kim J, Aoki F. Mechanisms of gene expression reprogramming during meiotic maturation and pre-implantation development. *J Mammal Ova Res.* 2004, 21, 89-96.
7. Kim J, Liu H, Tazaki M, [et al.]. Changes in histone acetylation during mouse oocyte meiosis. *J Cell Biol.* 2003, 162, 37-46.
8. Maalouf W, Alberio R, Campbell K. Differential acetylation of histone H4 lysine during development of in vitro fertilized, cloned and parthenogenetically activated bovine embryos. *Epigenetics.* 2008, 3, 199-209.
9. Sarmento O, Digilio L, Wang Y, [et al.]. Dynamic alterations of specific histone modifications during early murine development. *J Cell Sci.* 2004, 117, 4449-4459.
10. Wang Q, Yin S, Ai J, [et al.]. Histone deacetylation is required for orderly meiosis. *Cell Cycle.* 2006, 5, 766-774.
11. Endo T, Naito K, Aoki F, [et al.]. Changes in histone modifications during in vitro maturation of porcine oocytes. *Mol Reprod Dev.* 2005, 71, 123-128.
12. Huang J, Li T, Ding C, [et al.]. Insufficient histone-3 lysine-9 deacetylation in human oocytes matured in vitro is associated with aberrant meiosis. *Fertil Steril.* 2012, 97, 178-184.
13. Manosalva I, Gonzalez A. Aging alters histone H4 acetylation and CDC2A in mouse germinal vesicle stage oocytes. *Biol Reprod.* 2009, 81, 1164-1171.
14. Suo L, Meng Q, Pei Y, [et al.]. Effect of cryopreservation on acetylation patterns of lysine 12 of histone H4 (acH4K12) in mouse oocytes and zygotes. *J Assist Reprod Genet.* 2010, 27, 735-741.
15. Akiyama T, Nagata M, Aoki F. Inadequate histone deacetylation during oocyte meiosis causes aneuploidy and embryo death in mice. *Proc Natl Acad Sci U S A.* 2006, 103, 7339-7344.
16. Miao Y, Kikuchi K, Sun Q, Schatten H. Oocyte aging: cellular and molecular changes, developmental potential and reversal possibility. *Hum Reprod Update.* 2009, 15, 573-585.
17. Huang J, Yan L, Lei Z, [et al.]. Changes in histone acetylation during Postovulatory aging of mouse oocyte. *Biol Reprod.* 2007, 77, 666-670.
18. van den Berg I, Eleveld C, van der Hoeven M, [et al.]. Defective deacetylation of histone 4 K12 in human oocytes is associated with advanced maternal age and chromosome misalignment. *Hum Reprod.* 2011, 26, 1181-1190.
19. Gu L, Wang Q, Sun Q. Histone modifications during mammalian oocyte maturation: Dynamics, regulation and functions. *Cell Cycle.* 2010, 9, 1942-1950.
20. Liu H, Kim J, Aoki F. Regulation of histone H3 lysine 9 methylation in oocytes and early pre-implantation embryos. *Development.* 2004, 131, 2269-2280.
21. Zuccotti M, Merico V, Cecconi S, [et al.]. What does it take to make a developmentally competent mammalian egg? *Hum Reprod Update.* 2011, 17, 525-540.
22. Shi W, Haaf T. Aberrant methylation patterns at the two-cell stage as an indicator of early developmental failure. *Mol Reprod Dev.* 2002, 63, 329-334.
23. Dominko T, First N. Timing of meiotic progression in bovine oocytes and its effect on early embryo development. *Mol Reprod Dev.* 1997, 47, 456-467.
24. Ribas R, Taylor J, McCorquodale C, [et al.]. Effect of zona pellucida removal on DNA methylation in early mouse embryos. *Biol Reprod.* 2006, 74, 307-313.
25. Manosalva I, Gonzalez A. Aging changes the chromatin configuration and histone methylation of mouse oocytes at germinal vesicle stage. *Theriogenology.* 2010, 74, 1539-1574.
26. Fulka H, Langerova A, Barnetova I, [et al.]. How to repair the oocyte and zygote? *J Reprod Dev.* 2009, 55, 583-587.
27. Selesniemi K, Lee H, Muhlhauser A, Tilly J. Prevention of maternal aging-associated oocyte aneuploidy and meiotic spindle defects in mice by dietary and genetic strategies. *Proc Natl Acad Sci U S A.* 2011, 108, 12319-12324.