Spindle assembly checkpoint-related meiotic defect in oocytes from LT/Sv mice has cytoplasmic origin and diminishes in older females

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Abstract

The spindle assembly checkpoint (SAC) ensures proper segregation of chromosomes by delaying anaphase onset until all kinetochores are properly attached to the spindle microtubules. Oocytes from the mouse strain LT/Sv arrest at the first meiotic metaphase (MI) due to, as reported recently, enormously prolonged activity of the SAC. We compared the dynamics of cyclin B1–GFP degradation, the process which is a measure of the SAC activity, in chromosomal and achromosomal halves of LT/Sv oocytes. In chromosome-containing oocyte halves arrested at MI, cyclin B1–GFP was not degraded indicating active SAC. However, in the halves lacking chromosomes, which is a condition precluding the SAC function, degradation always occurred confirming that MI arrest in LT/Sv oocytes is SAC dependent. Transferring the germinal vesicle (GV) from LT/Sv oocytes into the enucleated oocytes from wild-type mice resulted in the progression through meiosis one, indicating that a SAC-activating defect in LT/Sv oocytes is cytoplasmic, yet can be rescued by foreign cytoplasm. These results may help to define the etiology of the human infertility related to the oocyte MI arrest, indicating the involvement of the SAC as likely candidate, and point to GV transfer as the possible therapy. Finally, we found that majority of oocytes isolated from old LT/Sv mice complete the first meiosis. Reciprocal transfers of the GV between the oocytes from young and old LT/Sv females suggest that the factor(s) responsible for the reversal of the phenotype in oocytes from old mice is located both in the GV and in the cytoplasm.

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Introduction

The final stage of oogenesis, meiotic maturation, comprises a set of events crucial for the process of fertilization and embryonic development. During this period, the oocyte reduces the number of chromosomes by progressing from the prophase of the first meiotic cell cycle to the metaphase of the second meiotic cell cycle. The complex control of this transition (Kubiak *et al.* 2008) includes the activity of the spindle assembly checkpoint (SAC), a mechanism that enables errorless segregation of the chromosomes in dividing cells by blocking the onset of anaphase when sensing the spindle or chromosome anomalies (Musacchio & Salmon 2007). The central element of the SAC pathway is monitoring the attachments of the microtubules to kinetochores – even one unattached kinetochore generates the signal inhibiting anaphase (Rieder *et al.* 1994, 1995). The functionality of the SAC was recently shown also during both the first (Tsurumi *et al.* 2004, Homer *et al.* 2005a,

2005b) and the second (Tsurumi *et al.* 2004) meiotic division in mammalian oocytes. However, the high incidence of chromosome segregation errors in mammalian female meiosis raises the question on the lower effectiveness of the SAC in oocytes (Vogt *et al.* 2008, Holt & Jones 2009).

The mouse mutant inbred strain LT/Sv exhibits a complex aberrant reproduction-related phenotype (Ciemerych *et al.* 2009). One of its features is the so-called oocyte metaphase I (MI) arrest as the majority of oocytes from LT/Sv strain instead finishing meiotic maturation stop their meiotic progression at the metaphase of the first meiotic division (Eppig 1978, Kaufman & Howlett 1986, Maleszewski & Yanagimachi 1995, Ciemerych & Kubiak 1998). Recent work revealed that MI arrest of LT/Sv oocytes is caused by the inability to timely inactivate the SAC (Hupalowska *et al.* 2008). Moreover, prolonged SAC activity also occurs during mitotic divisions of the early LT/Sv embryos contributing, together with meiotic defect, to the low reproductive

performance of this strain (Maciejewska *et al.* 2009). Several studies documented that MI arrest in the oocytes may also underlie some cases of human infertility (Eichenlaub-Ritter *et al.* 1995, Harrison *et al.* 2000, Bergère *et al.* 2001, Windt *et al.* 2001, Levran *et al.* 2002, Schmiady & Neitzel 2002, Combelles *et al.* 2003, Heindryckx *et al.* 2011). Therefore, LT/Sv oocytes may provide a valuable animal model to elucidate the etiology of similar disorders in humans and develop possible treatments.

As a consequence of the MI arrest, the LT/Sv mice ovulate the oocytes at this stage which, when fertilized, give rise to the triploid digynic embryos. Interestingly, the study by Speirs & Kaufman (1990) reported that the frequency of triploid embryos observed in LT/Sv females decreases with the age of the mothers. These authors suggested that this phenomenon may result from decreased frequency of MI arrest in LT/Sv oocytes in the older females; however, according to our knowledge, this hypothesis was never tested.

In this study, we analyzed the dynamics of cyclin B1 degradation, a process that is initiated only upon SAC inactivation and thus is an excellent way to study the status of the SAC activity (e.g. Clute & Pines 1999, Tsurumi *et al.* 2004, Homer *et al.* 2005a, 2005b). The analysis was performed in the chromosome-containing and chromosome-devoid fragments of oocytes from LT/Sv mice. We show that cyclin B1 degradation is inhibited in most cases when the chromosomes are present. However, in the absence of chromosomes, which is a condition precluding the SAC activity, the cyclin B1 degradation proceeds effectively, thus delivering another proof that MI arrest in LT/Sv oocytes results from enormously prolonged SAC activity. Nuclear transfer experiments involving LT/Sv oocytes and oocytes from wild-type mice show that the defect causing the prolonged activity of the SAC in the LT/Sv oocytes resides in the cytoplasm, not in the nucleus, of the prophase oocyte. Finally, we demonstrate that the frequency of MI arrest in LT/Sv oocytes drops significantly in the older females. This last finding is consistent with the report by Speirs & Kaufman (1990) who observed that the frequency of producing triploid embryos decreases in the old LT/Sv females.

Results

Dynamics of cyclin B1–GFP degradation proves the involvement of the SAC in the meiotic phenotype of LT/Sv oocytes

Recently, we have shown that interference with the Bub1 function greatly improves the rate of the progression through the first meiosis of LT/Sv oocytes, which shows that MI arrest is caused through sustained activity of the SAC (Hupalowska *et al.* 2008). To inquire further into the involvement of SAC in the meiotic phenotype of LT/Sv

mice, we performed the live-imaging of cyclin B1–GFP as the SAC activity inhibits cyclin B1 degradation (e.g. Clute & Pines 1999, Tsurumi *et al.* 2004, Homer *et al.* 2005a, 2005b). The oocytes were bisected to produce the chromosome-containing and the chromosome-devoid halves. Assuming that the LT/Sv defect is indeed related to the SAC activity, the cyclin B1–GFP level should be stabilized in the halves containing the chromosomes. In contrast, the degradation of cyclin B1–GFP should proceed in the chromosome-lacking oocyte halves due to the absence of the kinetochores, which provide the crucial element of the SAC pathway. In two experiments, the live imaging of cyclin B1–GFP was performed in eight chromosome-containing and seven chromosome-lacking halves as well as in three intact whole oocytes.

Among chromosome-containing halves, only two out of eight extruded the first polar body (PB1), which is consistent with the phenotype of LT/Sv oocytes. PB1 extrusion was accompanied with the rapid degradation of cyclin B1–GFP (Fig. 1), thus showing profile of cyclin B1 degradation typical for the wild-type oocytes undergoing the first meiotic division (Ledan *et al.* 2001, Homer *et al.* 2005a, 2005b). In the rest of chromosome-containing halves (six out of eight), PB1 extrusion did not occur, which indicated MI arrest. In such oocytes, cyclin B1–GFP showed unusual profile lacking rapid degradation but revealing rather the stabilization of the protein at the high level (Fig. 1). Accordingly, the cyclin B1–GFP degradation did not occur also in the whole (non-bisected) oocytes arrested at MI (three oocytes, data not shown). In contrast, in all chromosome-devoid oocyte halves ($n=7$), cyclin B1 was degraded rapidly (Fig. 1A and B). We have recently shown that degradation of cyclin B1–GFP in the chromosome-devoid oocyte halves is inhibited in the presence of MG132, which indicates that such degradation is proteasome dependent (Hoffmann *et al.* 2011). The difference between the behavior of the chromosome-containing halves (stabilization of cyclin B1–GFP) and the chromosome-devoid halves (rapid cyclin B1–GFP degradation) was statistically significant. These results confirm the involvement of the SAC in MI arrest in LT/Sv oocytes. The time of cyclin B1–GFP degradation in chromosome-devoid halves was earlier than in the chromosome-containing halves, which extruded PB1 (Fig. 1B). This results from the SAC activity maintained in the latter until the kinetochore–microtubule attachments are established.

The MI arrest in LT/Sv oocytes originates from a cytoplasmic defect

To inquire whether the primary defect, which prolongs SAC activity in LT/Sv oocytes, has cytoplasmic or nuclear origin, we performed the set of germinal vesicle (GV) transfer experiments. The GVs removed from LT/Sv

Figure 1 Imaging of the cyclin B1–GFP in the halves of LT/Sv oocytes undergoing meiotic maturation. (A) Fluorescence and DIC images. The upper and the lower panel show examples of the pairs of the oocyte halves obtained from the bisection of a single oocyte. Note that nuclear half (indicated by Hoechst staining) shown on the upper panel extruded PB1 (black arrow), whereas the nuclear half shown on the lower panel was arrested at MI. The images in 1 h intervals are shown starting at 15th min of imaging. (B) Semiquantitative analysis of the cyclin B1–GFP fluorescence signal. The mean values are shown for nuclear halves, which extruded PB1 (timing indicated by arrows; $n=2$), nuclear halves, which arrested at MI ($n=6$), and cytoplasmic halves ($n=7$). The data were collected in two independent experiments. The difference between the behavior of the chromosome-deprived halves (rapid cyclin B1–GFP degradation) and the chromosome-containing halves (stabilization of cyclin B1–GFP) is statistically significant ($P<0.05$; one-tailed Fisher exact probability test).

oocytes were transferred into enucleated GV stage OF1 oocytes and *vice versa*. Almost all oocytes that were manipulated to combine LT/Sv nucleus with OF1 cytoplasm extruded PB1. On the other hand, the majority of oocytes created by the transfer of wild-type GV into LT/Sv cytoplasm displayed arrest at the MI typical for LT/Sv oocytes (Table 1). These experiments clearly show that the defect underlying the MI arrest in LT/Sv oocytes has cytoplasmic origin.

The incidence of the oocyte MI arrest is greatly reduced in older LT/Sv females

We tested the meiotic progression *in vitro* in oocytes isolated from the LT/Sv females of different age. Whereas in the females up to 28 weeks old, only 23–31% of oocytes completed the first meiotic division; the proportion of such oocytes increased significantly in older females reaching 65% at the age 47–52 weeks (Fig. 2).

To see whether cytoplasm from a young oocyte can reestablish the MI arrest with an older nucleus (or *vice versa*), we have performed reciprocal GV transfer between LT/Sv oocytes isolated from young (age 8–22 weeks) or old (44–50 weeks) females (Table 2). The overall frequency of the oocytes that extruded PB1 was reduced in these conditions (compare, for e.g. Fig. 2 for the rate of PB1 extrusion in intact LT/Sv oocytes), nevertheless the difference between control young/young hybrid oocytes (composed of young GV and

young cytoplasm) and old/old oocytes (old GV and old cytoplasm) was retained and close to the statistical significance ($P=0.088$; Table 2). On the other hand, in the young/old hybrids as well as in the old/young ones, the proportion of PB1 extrusion was similar to that in the old/old oocytes (Table 2). This suggests that the factor(s) responsible for the increased rate of the PB1 extrusion in the oocytes from older mice is located both in their nucleus and in their cytoplasm.

Discussion

The inability to complete the first meiotic division by the majority of oocytes from the strain LT/Sv was recently attributed to the prolonged activity of the SAC (Hupalowska *et al.* 2008). In this study, the involvement of SAC in this specific meiotic phenotype was further confirmed by imaging cyclin B1–GFP. As previously shown in mouse oocytes undergoing meiosis I/meiosis II transition, the cyclin B1 degradation is inhibited until

Table 1 The progression through the first meiotic division in the hybrid oocytes produced by germinal vesicle transfer.

Composition of hybrid oocyte (GV:cytoplasm)	No. of oocytes	No. of oocytes extruding PB1 (%)
LT/Sv:OF1	33	24 (72.7) ^a
OF1:LT/Sv	26	8 (30.8) ^a

^aFrequency of oocytes extruding PB1 differs significantly between groups; $P<0.005$ (2×2 χ^2 test with Yates correction).

Figure 2 Oocytes from older LT/Sv females complete meiosis with higher efficiency. Bars represent mean $\pm$ S.E.M. Different letters indicate significant difference ($P < 0.05$; ANOVA with Bonferroni *post hoc* test). The number of females is shown in the parentheses. Mean numbers of tested oocytes/female in different age groups were as follows: 8–12 weeks, 15.9 oocytes (range 8–22); 15–19 weeks, 17.3 (12–32); 23–28 weeks, 17.2 (9–26); 32–36 weeks, 14.2 (9–21); and 47–52 weeks, 10.8 (6–19).

the SAC remains active (Tsurumi *et al.* 2004, Homer *et al.* 2005a, 2005b). Accordingly, in the few LT/Sv oocytes that complete first meiosis, the degradation of cyclin B1 occurs with similar timing and similar profile as in the typical mouse oocytes coinciding with the PB1 extrusion. On the other hand, in oocytes that were unable to undergo the first meiotic division, we never observed rapid and profound degradation of cyclin B1–GFP. Instead, the level of cyclin B1–GFP in such oocytes remained stabilized at the high level. This result is consistent with earlier, western blot-derived observation on the accumulation of cyclin B in the MI-arrested LT/Sv oocytes (Hampl & Eppig 1995). We show, however, that the removal of the chromosomes from the LT/Sv oocytes (by producing the oocyte halves lacking the chromosomes) invariably resulted with the rapid and efficient degradation of cyclin B1–GFP. As the kinetochores provide the crucial element of the SAC pathway, the removal of chromosomes (along with the kinetochores) breaks down the SAC, thus initiating cyclin B1 degradation. Efficient degradation of cyclin B1 occurring in LT/Sv oocytes upon chromosome removal provides clear evidence that MI arrest is mediated by the activity of SAC supporting our previous report (Hupalowska *et al.* 2008). These results also show that the proteolytic mechanism that acts downstream of the SAC and executes cyclin B1 degradation functions efficiently in LT/Sv oocytes.

The nature of the SAC-affecting defect in LT/Sv oocytes remained unclear until now. Using nuclear transfer, we show that the hybrid oocytes composed of LT/Sv GV and wild-type cytoplasm do not suffer from the MI arrest

whereas the hybrid oocytes containing wild-type GV within the LT/Sv cytoplasm do arrest at MI. These results show that the defect causing prolonged SAC activity in LT/Sv oocytes is cytoplasmic. Assuming that the prolonged checkpoint activity in LT/Sv oocytes reflects SAC-sensed unidentified anomalies within the MI spindle, these results suggest that the primary cause of the MI arrest may lie in the spindle components recruited from the cytoplasm. In this context, it is noticeable that LT/Sv oocytes were reported to exhibit unusual organization of centrosomes at the prophase stage (Albertini & Eppig 1995). On the other hand, previous studies did not reveal abnormal morphology of the MI spindle in LT/Sv oocytes 7 h after GV breakdown (GVBD; Ciemerych & Kubiak 1998), i.e. at the time when oocytes from other mouse strains already extrude the PB1s or are close to start this process (Polanski 1997). Therefore, another possibility explaining LT/Sv phenotype shall assume that spindle formation in LT/Sv oocytes is unperturbed and the MI arrest results from the lesion within the SAC pathway itself. According to our results, future research exploring this possibility should concentrate on the putative SAC components, which were proven to reside in the cytoplasm rather than nucleus of the prophase oocyte.

Several recent studies showed that some cases of unexplained infertility in humans result from arrest of the oocytes at the MI stage (Eichenlaub-Ritter *et al.* 1995, Harrison *et al.* 2000, Bergère *et al.* 2001, Windt *et al.* 2001, Levrant *et al.* 2002, Schmiady & Neitzel 2002, Combelles *et al.* 2003, Heindryckx *et al.* 2011). It is obvious that the SAC represent a potent mechanism arresting the cell at metaphase. A number of factors may affect the spindle structure, thus keeping the SAC active; moreover, the prolonged SAC activity may reflect a defect in the SAC pathway itself. Thus, a lot of scenarios may lead to maintain the SAC active resulting in the metaphase arrest. Therefore, the SAC activity, which was shown to block oocytes at MI in LT/Sv mice (Hupalowska *et al.* 2008; this study), shall be considered as major candidate responsible for the cases of MI arrest also in human oocytes. The cases of MII arrest in fertilized human eggs were also reported (Levrant *et al.* 2002). These may result from defective sperm being unable to

Table 2 The progression through the first meiotic division in the hybrid LT/Sv oocytes produced by germinal vesicle transfer between oocytes from young and old females.

Composition of hybrid LT/Sv oocyte (GV:cytoplasm)	No. of oocytes	No. of oocytes extruding PB1 (%)
Young:old	36	9 (25%)
Old:young	34	8 (23.5%)
Old:old	33	9 (27.3%)
Young:young	35	4 (11.4%)

The age of the young females was 8–22 weeks and the old ones 44–50 weeks.

deliver activating stimulus or from oocyte defect being unable to respond properly to sperm penetration. Notably, as the SAC was shown to function in oocytes also during the second meiotic cycle (Tsurumi *et al.* 2004, Homer *et al.* 2005a), its involvement in at least some cases of human infertility related to the oocyte MI arrest cannot be excluded.

The GV transfer or the cytoplasm transfer represents the possible treatment of human infertility related to oocyte defect (Cohen *et al.* 1997, Krey *et al.* 2001), although the safety of these procedures still needs full confirmation (Levy *et al.* 2004). We show that transfer of GV from LT/Sv oocyte into the wild-type cytoplasm rescues the meiotic progression of LT/Sv nucleus. This points to GV transfer (or perhaps transfer of the donor cytoplasm from healthy oocyte into patient's oocyte) as a possible effective treatment of the cases of recurrent human oocyte MI arrest, especially in the light of unsuccessful attempts of an alternative therapy (Heindryckx *et al.* 2011).

We show dramatic decrease in the incidence of oocytes blocked by the SAC at MI in oocytes from aged LT/Sv females. This may mean that the primary cytoplasmic defect, which activates the SAC in LT/Sv oocytes, disappears with the female age. Alternatively, it might be possible that in older females such primary defect persists, and the low frequency of MI arrest results, paradoxically, from decreased quality of the oocytes in older females, which is a well-known phenomenon. One could consider at least three plausible explanations linking such a maternal age-dependent phenotype of LT/Sv oocytes with age-dependent deterioration of oocytes. Some previous studies reported the decreased level of the SAC proteins or their transcripts in the oocytes or ovaries of older females in mice (Baker *et al.* 2004, Pan *et al.* 2008) and in humans (Steuerwald *et al.* 2001), suggesting the lower effectiveness of the SAC during mammalian female meiosis and thus implicating defective SAC as one of the possible sources of age-related aneuploidy (Holt & Jones 2009). Therefore, one could assume that the primary cytoplasmic defect persists in the oocytes from old LT/Sv females; however, the weakened SAC is unable to maintain the MI arrest. The weakness of such hypothesis is, however, that the mouse study provided no proof on the decreased SAC activity in the oocytes from old females (Duncan *et al.* 2009). Instead, several recent studies show a failure in the chromosome cohesion in the oocytes from old females, which becomes a leading hypothesis elucidating maternal age-related aneuploidy (Liu & Keefe 2008, Chiang *et al.* 2010, 2011, Lister *et al.* 2010). Therefore, the second possible explanation implies that in oocytes from old LT/Sv females, the chromosomes lose their cohesion, which results in their separation and possibly induce the PB1 extrusion. Lastly, it is also possible that deterioration of the oocyte quality in the older females could affect the cytoskeletal factor (CSF) activity, which

normally blocks the oocyte at the MI stage. Hirao & Eppig (1997) previously demonstrated that the CSF activity is required for the maintenance of LT/Sv oocytes at the permanent MI arrest. Consistently, any failure in the CSF activity, which could eventually occur in the oocytes from old LT/Sv females, would result in the breakdown of the MI arrest and completion of the first meiosis.

The experiments with the reciprocal transfer of GV between the oocytes isolated from LT/Sv females of different age give some indications on the nature of the change of the phenotype in oocytes from old LT/Sv mice (Table 2). As demonstrated in Table 2, control hybrids reconstituted from 'old' oocytes extrude PB1 with higher efficiency than the control hybrids reconstituted from 'young' oocytes. On the other hand, the mixed hybrids composed of young GV and old cytoplasm (young GV/old cytoplasm) as well as the hybrids with reversed combination (old GV/young cytoplasm) have the maturation rate similar to the reconstituted 'old' oocytes. Thus, the factor responsible for the elevated frequency of PB1 extrusion of 'old' LT/Sv oocytes seems to be located both in the GV and in the cytoplasm. We, on the other hand, know that the primary defect responsible for the MI arrest in the young LT/Sv oocytes is purely cytoplasmic (Table 1). As mentioned earlier, one of the possible explanations of the change of the phenotype in old LT/Sv oocytes comprises the retraction of this primary cytoplasmic defect responsible for MI arrest. If this primary defect, however, would retract in the 'old' oocytes, we should see the increase in PB1 extrusion only in the 'young GV/old cytoplasm' hybrids, but not in the 'old GV/young cytoplasm' ones. The obtained results, therefore, favor the alternative hypothesis that in the oocytes from old LT/Sv females, the primary defect still persists, but, due to some other changes in old oocytes, it cannot be expressed phenotypically. Moreover, we should also exclude the possibility that the elevated frequency of the PB1 extrusion in the 'old' oocytes could be related to the weakening of the cohesins as, in that case, we should see increased proportion of PB1 extrusion in the 'old GV/young cytoplasm' combination but not in the young GV/old cytoplasm' ones.

Therefore, from the possible explanations for the increased frequency of completion of the first meiosis in oocytes from old LT/Sv females presented earlier, we should consider as the most likely ones the two: that in the oocytes from old females either the SAC or the CSF weakens. Notably, the immunofluorescence studies revealed that crucial CSF component Emi2 (Shoji *et al.* 2006) is located in the GV of mouse oocytes (Suzuki *et al.* 2010) and the GV localization was demonstrated also for the key SAC proteins Mad2 (Wang *et al.* 2009), BubR1 (Wei *et al.* 2010) and Bub3 (Li *et al.* 2009). It is possible that these proteins are also located in the cytoplasm as even slightly elevated amount of the protein in the GV may give the distinct immunofluorescent signal when compared with the cytoplasm.

We have shown such relationship previously for the PLK1, which gave distinct GV-associated staining; however, the western blots of GV-containing and GV-deprived oocyte halves revealed that it is also abundant in the cytoplasm (Pahlavan *et al.* 2000). Therefore, both CSF-related and the SAC-related explanations for the change of the phenotype in the old LT/Sv oocytes seem to be at present equally possible.

Materials and Methods

Animals

The mice from LT/Sv strain as well as the wild-type mice from the stock OF1 were used. The experiments were approved by the Local Ethics Committee no. 1 in Krakow, Poland, and Regierungspräsidium in Freiburg, Germany, according to the European Union Council Directive 86/609/EEC of 24 November 1986.

Oocyte collection, micromanipulation, and culture

Mouse oocytes at the GV stage (GV oocytes) were isolated from the ovaries of 2- to 5-month-old females of OF1 or LT/Sv mice, except experiments designed to test the effect of age on the meiotic phenotype of LT/Sv mice in which the cohorts of mice at specified age were used. Oocytes were cultured as described previously (Polanski *et al.* 2005) in the M2 medium (Sigma–Aldrich) under mineral oil (Sigma–Aldrich). GV transfer was performed as described previously (Polanski *et al.* 2005) in the M2 medium supplemented with the dbcAMP (Sigma–Aldrich) to prevent meiosis resumption before finishing the micromanipulation procedure. Rinsing out dbcAMP was considered as the start of the culture of micromanipulated oocytes.

Dynamics of cyclin B1–GFP degradation

Oocytes from LT/Sv mice were cultured for 2 h and these which resumed meiosis (indicated by GVBD) were injected with cyclin B1–GFP RNA (Ledan *et al.* 2001). After 1 h of the culture, the zona pellucida was removed from injected oocytes by the pronase treatment (Polanski 1997) and oocytes were transferred into the cytochalasin B containing medium in which they were bisected into two halves as described previously (Tarkowski 1977, Hoffmann *et al.* 2006). To discriminate between chromosome-containing and chromosome-devoid oocyte halves, they were then labeled for 30 min at 37 °C with vital Hoechst (bisbenzimidazole, H 33258, Sigma–Aldrich, 1 µg/ml) and finally cultured in the M2 medium on the stage of inverted Zeiss Axiovert200M microscope for live imaging. At the start of the culture, the image using the filter appropriate for vital Hoechst was taken to identify the halves containing the chromosomes and those which contained the cytoplasm only. During the further culture, the images were captured every 15 min at GFP and DIC channels. The intensity of the fluorescence generated by GFP was quantified on the acquired images using Adobe Photoshop. In each image analyzed, the virtual circular frame with the diameter slightly less than the diameter of the oocyte half was created. This frame was superimposed over the oocyte

half and the mean luminosity of the marked area was measured using ‘histogram’ function. Then, the same frame was moved to cover the next oocyte half followed by measurement. Using the same frame, all oocyte halves on all the images within a whole single experiment were analyzed. The same frame was also superimposed over the area where no oocyte halves were present (always the left lower corner of the image) to measure the background on the each image. The background value was then subtracted from the value obtained for each oocyte half analyzed on a given image.

The effect of female age on the meiotic progression of LT/Sv oocytes

Oocytes were isolated in the M2 and after removing the cumulus cells placed in the culture for 18 h. At the end of the culture, the number of oocytes that extruded the PB1 was determined.

Statistical analysis

The data were analyzed using 2×2 χ^2 test (in some cases, the Fisher exact probability test was used as more appropriate for the analysis of samples with low numbers) or one-way ANOVA. In the latter case, the percentage values were first processed for angular arcsine transformation, and after running ANOVA, the differences between groups were determined using Bonferroni *post hoc* test. $P < 0.05$ was considered statistically significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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