EMBO reports

Birth of mice from meiotically arrested spermatocytes following biparental meiosis in halved oocytes

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Revision 0

Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Ogonuki et al developed a new technique using primary spermatocyte-injected oocytes for offspring production. They examined chromosome segregation error in biparental meiosis using spermatocyte-injected oocytes. They showed that artificially reducing ooplasmic volume rescued highly error-prone chromosome segregation by preventing sister separation in biparental meiosis. Their live-imaging analysis demonstrated that erroneous chromosome segregation derived from univalent-like chromosomes followed by predivision of sister chromatids during prometaphase I in biparental meiosis. They showed that the birth rate was improved using halved oocytes. Furthermore, they showed that production of offspring was successful using spermatocyte from azoospermic mice.

Overall data are convincing and the manuscript addresses important questions. The data was produced in a technically high level. Presented data are sufficient to support conclusions of the authors, and further provide a significant insight into application to production of offspring for azoospermia animals. Thus, the manuscript could be open for the fields and are supposed to deserve publication, if they could address following minor concerns.

Fig1A, Line 117
This is an amazing experiment to set up biparental meiosis using spermatocyte nuclei. Since spermatocytes are in different stages during progression through meiotic prophase, some of them (late pachytene) should yield crossover but others (before mid-pachytene) are yet to complete recombination. Thus, whether donor paternal chromosomes have bivalents or univalents depends on which stage spermatocytes derived from. The authors should describe how spermatocytes were picked up for injection and whether they used a particular stage of spermatocytes.

Line 159-160
The authors stated that paternal chromosomes are susceptible to errors in ooplasm-hosted biparental meiosis. This is nice demonstration to trace the origin of separated chromatids. In Fig2C right graph, 1 to 2 paternal chromosomes showed misalignment. It is unclear whether premature separation is biased to any particular paternal chromosome, eg XY ? The authors should discuss more about it.

Line 176-177
The authors stated that most of errors were preceded by premature separation of bivalent chromosomes into univalent-like structures. This implies that premature separation of bivalent chromosomes happens prior to anaphase onset. Does this depend on spindle force? Or is cohesion intrinsically fragile in donor spermatocyte chromosomes? The authors should discuss more about it.

Fig3E,

The authors depicted that in normal sized oocytes, univalent-like chromosomes undergo predivision at anaphase. This is somewhat too simplified, because Fig3B shows that a certain population exhibits nondisjunction. This model and description should be corrected to fit the data they demonstrated. If sister segregation at anaphase is predominant, I wonder what happens to sister kinetochore mono-orientation and sister centromeric protection in such univalent-like chromosomes. It would be nice to show centromeric proteins MEIKIN, SGO2 in donor spermatocyte chromosomes versus those of oocyte to examine centromeric cohesion. The authors should clarify this issue.

Line296-294
What do the authors mean by the sentence " It is known that sex chromosomes are prepared to undergo meiosis later than autosomes."?

2. Significance:

Significance (Required)

The manuscript will provide biological significance for the reproduction fields. There are two major biological significances : They addressed the mechanism of erroneous chromosome segregation in biparental meiosis. They showed that biparental meiosis using spermatocyte-injected oocytes can be applied to production of offspring of azoospermic mice, which would have great impact on reproductive biology field. The data was produced with their high level of technique.

Referee Cross-commenting

I agree to the point described in Reviewer #3's Main points2. It would be better to see SAC proteins.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary:

Previously, the team has shown that primary spermatocyte nucleus can undergo meiosis when transplanted into immature oocytes, and later obtained normal mice from the fertilized oocytes (Zygotes 1997, PMID: 9276513; PNAS 1998, PMID: 9576931). However, the efficiency was quite low (~ 1%) due to chromosome aberration, thus not feasible for basic/clinical research applications. In this study, Ogonuki et al., extrapolated from the recent study showing the reduction of the ooplasm ameliorate the error of chromosome segregation during meiosis (Dev Cell 2017, PMID: 28486131), injected the spermatocyte nucleus into the half-sized GV oocytes, and succeeded to obtain live murine pups with a high incidence (the birth rate improved from 1% with full-sized oocytes to 19% with half-sized oocytes). Further, through detailed observation with high-resolution 3D live imaging, the authors clarified that the misalignment of paternal chromosomes could be ameliorated by reducing the volume of ooplasm. Finally, the authors applied this technology and obtained live pups from azoospermic mice, suggesting the potential application in human infertility treatment.

Major comments:

This is a great study combining the expertise on both sperm and oocytes. The experiments are well designed and performed. The key conclusions are convincing.

Line 228. The authors claimed that all the pups born following the injection of wild-type or mutant spermatocytes grew into fertile adults.

Because the authors tested 3 males from wt spermatocytes (line 197), the above sentence should be rephrased.

The authors found one XXY male among the three male mice from wt spermatocytes. Was the XYY male mouse fully fertile without XY/XYY mosaicism?

How many females and males were obtained from wt spermatocytes?

Minor comments:

The authors clearly showed the technique can be applied to rescue the spermatogenic arrest. The readers would appreciate if the authors include any unsuccessful cases.

To prevent sex-chromosome aberration, are there any potential markers for selecting most developed spermatocytes?

2. Significance:

Significance (Required)

One in six couples suffers from infertility, and 70-90% of male infertility cases are related to defects in spermatogenesis. Clinically, intracytoplasmic injection of sperm is common, but it is not applicable to men who lack haploid germ cells. Injection of primary spermatocyte nucleus can give pups but the efficiency was poor (~1%, PNAS 1998, PMID: 9576931). In the present study, by using halved oocytes as recipient, the authors improved the efficiency from 1% to 19%. With the great improvement, they further obtained healthy fertile offspring from the male mice genetically lacking haploid cells. This approach opens up the window for the infertile patients suffering from spermatogenic arrest.

The reviewer's field of expertise: knockout mice, male infertility, spermatogenesis, sperm function, fertilization.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Less than 1 month

Review #3

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

In mice, failures in conducting meiosis during spermatogenesis can be rescued by injecting prophase I male chromosomes into oocytes, to allow them to undergo the two meiotic divisions within the oocyte, together with the chromosomes of the oocyte. However, segregations are highly error prone and rarely lead to a live birth when the resulting embryos are reimplanted into foster mothers. In this study, the authors show that segregation errors in meiosis I oocytes harboring both male and female chromosomes are mainly affecting the male chromosome set. Most errors are due to precocious segregation of sister chromatids in unpaired male chromosomes (univalents). A delay in alignemnt of male chromosomes compared to female chromosomes was also observed. Reducing the volume of the oocyte cytoplams to half leads to a signifncant reduction in the errors occuring, and hence, a significant increase in successful birth after re-implantation. Excitingly, with this technique, live births were obtained from male mice with a spermatogenic arrest phenotype.

Main points:

1)The authors conclude that halving the oocyte cell size is helping in proper segregation of male meiosis I chromosomes in the cytoplasm of meiosis I oocytes. It is also possible that the experimental procedure involved in removing half of the cytoplasm is promoting proper segregation for some unknown reason. The authors should include a condition where half of the cytoplasm is aspirated but then put back again, so oocytes have the same volume as before but the cytoplasm underwent the same treatment as in the halved oocytes. Also, increasing the cytoplasm volume of the oocyte should not lead to a better segregation of male chromosomes but make things worse, have the authors checked for that?

2)The authors mention that male chromosomes align with a delay, compared to the female chromosomes. Does this delay depend on activation of error correction, or the spindle asembly checkpoint? Is it possible that dilution of factors required for checkpoint control and hence, assuring proper chromosome segregation, are

the reason for error prone segregation in oocytes harboring twice the amount of chromosomes? If yes, have the authors stained for SAC proteins at the kinetochores? Maybe slight overepxression of the SAC protein were sufficient to rescue male meiotic divisions in the oocyte- have the authors tested this hypothesis?

3) The authors state that male chromosomes have a hard time segregating in the hugh cytoplasm of the oocytes. Maybe it is not the fact that the chromosomes came from a male pronucleus, but this is just a manner of double the chromosomes that have to be segregated in the oocyte cytoplams. How do male chromosomes behave in enucleated oocytes undergoing meiosis I? Conversely, if female chromosomes coming from another oocyte are injected into the recipient oocyte instead of ale chromosomes, are those segregating correctly, or the delay in chromosome alignment and error rate comparable to the situation when the additional chromosome set comes from the male?

4) In the rescue of mice with spermatogenic arrest the authors find aneuploidies of sex-chromosomes in the off-spring, not of autosomes. To my best of knowledge, autosome aneuploidies are not viable in the mouse, hence this result does not indicate that sex-chromosomes are the main source of aneuploidies. Nevertheless, it is attractive to speculate that aneuploidies are mainly due to sex chromosomes, because the oocyte is not prepared to segregate a male sex-chromosome bivalent. The authors should determine whether the segregation errors in meiosis I in oocytes harboring the additional male chromosome set concern mainly the male sex-chromosomes, by doing Fish analysis after meiosis I.

2. Significance:

Significance (Required)

This study is very interesting and of high significance, and very well executed. I think the study can go much further as far as mechanistic insights are concerned, only requiring techniques and tools that the authors have at their disposition.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 3 and 6 months

Full Revision

Manuscript number: RC-2021-01059
Corresponding author(s): Atsuo Ogura and Tomoya S. Kitajima

1. General Statements [optional]

We have revised the paper by performing all the necessary experiments as suggested by the reviewers. In the revised files, all changes to the text are indicated in red. As the number of figures increased, we have reorganized all the figures as follows:
Figure 1 (no change)
Figure 2 (no change)
Figure 3 (3E was modified)
Figure 4 (new figure)
Figure 5 (from Fig. 4)
Figure 6 (from Fig. S3)
Figure S1 (new figure)
Figure S2 (from Fig. S1)
Figure S3 (from Fig. S2)
Figure S4 (no change)
Figure S5 (new figure)

2. Point-by-point description of the revisions

Reviewer 1

Ogonuki et al developed a new technique using primary spermatocyte-injected oocytes for offspring production. They examined chromosome segregation error in biparental meiosis using spermatocyte-injected oocytes. They showed that artificially reducing ooplasmic volume rescued highly error-prone chromosome segregation by preventing sister separation in biparental meiosis. Their live-imaging analysis demonstrated that erroneous chromosome segregation derived from univalent-like chromosomes followed by predivision of sister chromatids during prometaphase I in biparental meiosis. They showed that the birth rate was improved using halved oocytes. Furthermore, they showed that production of offspring was successful using spermatocyte from azoospermic mice.

Overall data are convincing and the manuscript addresses important questions. The data was produced in a technically high level. Presented data are sufficient to support conclusions of the authors, and further provide a significant insight into application to production of offspring for azoospermia animals. Thus, the manuscript could be open for the fields and are supposed to deserve publication, if they could address following minor concerns.

>> REPLY: We are gratified to know that Reviewer 1 appreciated the significance of our study

and has evaluated it highly. We are also grateful for the constructive comments on our manuscript. Based on these comments, we have modified the manuscript as detailed below.

Comment #1 (Fig1A, Line 117)
This is an amazing experiment to set up biparental meiosis using spermatocyte nuclei. Since spermatocytes are in different stages during progression through meiotic prophase, some of them (late pachytene) should yield crossover but others (before mid-pachytene) are yet to complete recombination. Thus, whether donor paternal chromosomes have bivalents or univalents depends on which stage spermatocytes derived from. The authors should describe how spermatocytes were picked up for injection and whether they used a particular stage of spermatocytes.
>> REPLY: The primary spermatocytes we used were in the later pachytene to diplotene stages with bivalent chromosomes. They comprise the population of the largest spermatogenic cells (18–20 μm in diameter) and have a distinct nuclear membrane. We confirmed that we can pick up these late prophase spermatocytes at nearly 100% accuracy by observing the condensed tetrad chromosomes after injecting them into MII stage oocytes. This chromosome morphology was consistent with that of spermatocyte chromosomes induced to enter the MI stage by okadaic acid treatment (Cobb et al. Dev Biol 205: 49–64, 1999). These descriptions and a relevant photograph were added to the revised version (Lines 117–121 and Fig. 1B).

Comment #2 (Line 159-160)
The authors stated that paternal chromosomes are susceptible to errors in ooplasm-hosted biparental meiosis. This is nice demonstration to trace the origin of separated chromatids. In Fig2C right graph, 1 to 2 paternal chromosomes showed misalignment. It is unclear whether premature separation is biased to any particular paternal chromosome, eg XY? The authors should discuss more about it.
>> REPLY: We agree that this is important for a correct understanding of the cause of sex chromosome-biased aberrations in spermatocyte-derived offspring. For this purpose, we successfully developed a multicolor FISH (fluorescence *in situ* hybridization) technique for mouse MII oocytes. This technique clearly identifies each autosome and sex chromosome. By analyzing spermatocyte-injected oocytes at the MII stage, we concluded that chromosomal abnormalities occurred in both autosomes and X chromosome (Lines 294–307, Fig. S5). Therefore, it was most likely that only embryos with normal autosomes survived to term and, consequently, the pups showed only sex chromosome abnormalities, if any.

Comment #3 (Line 176-177)
The authors stated that most of errors were preceded by premature separation of bivalent chromosomes into univalent-like structures. This implies that premature separation of bivalent chromosomes happens prior to anaphase onset. Does this depend on spindle force? Or is cohesion intrinsically fragile in donor spermatocyte chromosomes? The authors should discuss more about it.
>> REPLY: We cannot distinguish these possibilities from our results. In the manuscript, we added a discussion point: “our live imaging demonstrated that premature bivalent separation

occurred predominantly in spermatocyte-derived chromosomes, preceding chromosome segregation errors. The spermatocyte chromosomes might be intrinsically more error-prone than oocyte chromosomes. Alternatively, the biochemical environment of the oocyte cytoplasm or spindle forces might have selectively promoted the separation of the spermatocyte chromosomes. The lack of the SAC activator MAD2 and delayed alignment of the spermatocyte chromosomes (Figures 2B,2C, 4C) may reflect spermatocyte-chromosome-specific difficulties in biparental meiosis." (Lines 338–346).

Comment #4 (Fig.3E)
The authors depicted that in normal sized oocytes, univalent-like chromosomes undergo predivision at anaphase. This is somewhat too simplified, because Fig3B shows that a certain population exhibits nondisjunction. This model and description should be corrected to fit the data they demonstrated. If sister segregation at anaphase is predominant, I wonder what happens to sister kinetochore mono-orientation and sister centromeric protection in such univalent-like chromosomes. It would be nice to show centromeric proteins MEIKIN, SGO2 in donor spermatocyte chromosomes versus those of oocyte to examine centromeric cohesion. The authors should clarify this issue.
>> REPLY: We thank the reviewer for this constructive comment. We revised the model in Fig. 3E to show the causes of chromosome nondisjunction. Concerning the issues of sister kinetochore mono-orientation and sister centromeric protection, an antibody for SGO2 was available, but unfortunately the previously published antibody against MEIKIN (Kim et al. *Nature* 517: 466–471, 2015) does not work in oocytes (Maier et al. *Dev. Cell* 56: 2192–2206, 2021). Therefore, we used an antibody for PLK1, a Polo-like kinase enriched for kinetochores in a MEIKIN-dependent manner (Kim et al. *Nature* 517: 466–471, 2015). Our immunostaining analysis clearly revealed that both SGO2 and PLK1 were localized at the kinetochores of spermatocyte-derived chromosomes (Fig. 4). Thus, the predivision of spermatocyte-derived chromosomes is unlikely to be explained by defects in the recruitment of these proteins (Lines 195–205, Fig. 4).

Comment #5 (Line292-294)
What do the authors mean by the sentence " It is known that sex chromosomes are prepared to undergo meiosis later than autosomes."?
>> REPLY: We apologize for this unclear expression. As our multicolor FISH analysis revealed that chromosomal aberrations occurred in both autosomes and sex chromosomes, the paragraph containing this sentence has been omitted in the revised version.

(Significance (Required)):
The manuscript will provide biological significance for the reproduction fields. There are two major biological significances : They addressed the mechanism of erroneous chromosome segregation in biparental meiosis. They showed that biparental meiosis using spermatocyte-injected oocytes can be applied to production of offspring of azoospermic mice, which would have great impact on reproductive biology field. The data was produced with their high level of technique.

>> REPLY: We appreciate the reviewer's positive comments on two major biological significances of our study. We hope that our information would provide invaluable clues for better understanding of the mechanisms of meiosis and further advancements of reproductive technologies in humans and animals.

Referee Cross-commenting
I agree to the point described in Reviewer #3's Main points2. It would be better to see SAC proteins.
>> REPLY: Please see the reply to Reviewer #3.

Reviewer 2

Summary:
Previously, the team has shown that primary spermatocyte nucleus can undergo meiosis when transplanted into immature oocytes, and later obtained normal mice from the fertilized oocytes (Zygotes 1997, PMID: 9276513; PNAS 1998, PMID: 9576931). However, the efficiency was quite low (~ 1%) due to chromosome aberration, thus not feasible for basic/clinical research applications. In this study, Ogonuki et al., extrapolated from the recent study showing the reduction of the ooplasm ameliorate the error of chromosome segregation during meiosis (Dev Cell 2017, PMID: 28486131), injected the spermatocyte nucleus into the half-sized GV oocytes, and succeeded to obtain live murine pups with a high incidence (the birth rate improved from 1% with full-sized oocytes to 19% with half-sized oocytes). Further, through detailed observation with high-resolution 3D live imaging, the authors clarified that the misalignment of paternal chromosomes could be ameliorated by reducing the volume of ooplasm. Finally, the authors applied this technology and obtained live pups from azoospermic mice, suggesting the potential application in human infertility treatment.

Major comments:
This is a great study combining the expertise on both sperm and oocytes. The experiments are well designed and performed. The key conclusions are convincing.
>> REPLY: We are gratified to know that Reviewer 1 appreciated the significance of our study and has evaluated it highly. We are also grateful for the constructive comments on our manuscript. Based on these comments, we have modified the manuscript as detailed below.

Major Comment #1
Line 228. The authors claimed that all the pups born following the injection of wild-type or mutant spermatocytes grew into fertile adults.
Because the authors tested 3 males from wt spermatocytes (line 197), the above sentence should be rephrased.
>> REPLY: We agree with this point. Unfortunately, we euthanized the remaining spermatocyte-derived pups at birth after confirming their normality in appearance and respiration (please see reply to Minor Comment #2 below). We rewrote the phrase: "all 11 spermatocyte-derived pups

(three wild-type-derived and eight knockout-derived) nursed by the foster mothers grew into fertile adults." (Lines 279–280).

Major Comment #2
The authors found one XXY male among the three male mice from wt spermatocytes. Was the XYY male mouse fully fertile without XY/XYY mosaicism?
>> REPLY: We are sorry for not explaining the reproductive performance of the XYY male mouse in detail. This mouse could only impregnate one out of ten female mice, so was subfertile. According to Obata et al., (PNAS 2008), such XYY male mice are not always completely infertile. Nevertheless, we cannot exclude the possibility of XY/XYY mosaicism in this mouse, although we karyotyped more than 20 spleen cells. These results were incorporated into the revised version (Lines 284–288).

Major Comment #3
How many females and males were obtained from wt spermatocytes?
>> REPLY: Unfortunately, we had to euthanize the first three batches of pups (14 pups from the first three experiments in Supplemental Table S3) derived from wild-type spermatocytes because of limited animal room space. In the last two experiments, we obtained three male mice and they were used for karyotyping and fertility testing. Therefore, we do not know the exact numbers of male and female mice obtained from wild-type spermatocytes. We would appreciate the reviewers' kind understanding of this limitation.

Minor Comment #1
The authors clearly showed the technique can be applied to rescue the spermatogenic arrest. The readers would appreciate if the authors include any unsuccessful cases.
>> REPLY: As suggested by the reviewer, we conducted spermatocyte microinjection experiments using D1Pas1 gene knockout mice, which show spermatocyte arrest before the late pachytene stage. We successfully constructed two-cell embryos using these knockout spermatocytes, but no pups were born following embryo transfer, probably because we microinjected spermatocytes before the mid-pachytene stage, or they were somewhat damaged by cell death. We added this result in the revised version (Lines 268–276; Table S3).

Minor Comment #2
To prevent sex-chromosome aberration, are there any potential markers for selecting most developed spermatocytes?
>> REPLY: As we replied to Comment #2 of Reviewer 1, we examined whether sex-chromosome-biased aberrations occurred in oocytes by multicolor FISH using spermatocyte-derived MII oocytes. As a result, we could conclude that chromosome aberrations randomly occurred in both autosomes and sex chromosomes within spermatocyte-injected oocytes (Lines 294–307, Fig. S5). Therefore, it is most likely that only embryos with normal autosomes survived to term and the living pups showed only sex chromosome abnormalities, if any. Furthermore, transfer of MI oocyte karyoplasts, instead of spermatocytes, into recipient MI oocytes also caused chromosomal aberrations (Lines 218–228, Fig. S1). Based on these

findings, we assume that use of most developed spermatocytes would not improve the chromosome integrity of spermatocyte-injected oocytes. We would appreciate the reviewer for kindly understanding this issue.

(Significance (Required)):
One in six couples suffers from infertility, and 70-90% of male infertility cases are related to defects in spermatogenesis. Clinically, intracytoplasmic injection of sperm is common, but it is not applicable to men who lack haploid germ cells. Injection of primary spermatocyte nucleus can give pups but the efficiency was poor (~1%, PNAS 1998, PMID: 9576931). In the present study, by using halved oocytes as recipient, the authors improved the efficiency from 1% to 19%. With the great improvement, they further obtained healthy fertile offspring from the male mice genetically lacking haploid cells. This approach opens up the window for the infertile patients suffering from spermatogenic arrest.
>> REPLY: We appreciate the reviewer's supportive comments in relation to the current status of the human male-factor infertility. We hope that our information would provide invaluable clues for human clinical research aiming to develop treatments for meiosis-related male infertility.

Reviewer 3

In mice, failures in conducting meiosis during spermatogenesis can be rescued by injecting prophase I male chromosomes into oocytes, to allow them to undergo the two meiotic divisions within the oocyte, together with the chromosomes of the oocyte. However, segregations are highly error prone and rarely lead to a live birth when the resulting embryos are reimplanted into foster mothers. In this study, the authors show that segregation errors in meiosis I oocytes harboring both male and female chromosomes are mainly affecting the male chromosome set. Most errors are due to precocious segregation of sister chromatids in unpaired male chromosomes (univalents). A delay in alignemnt of male chromosomes compared to female chromosomes was also observed. Reducing the volume of the oocyte cytoplams to half leads to a signifncant reduction in the errors occuring, and hence, a significant increase in successful birth after re-implantation. Excitingly, with this technique, live births were obtained from male mice with a spermatogenic arrest phenotype.

>> REPLY: We are gratified to know that Reviewer 1 appreciated the significance of our study and has evaluated it highly. We are also grateful for the constructive comments on our manuscript. Based on these comments, we have modified the manuscript as detailed below.

Main Point
1)The authors conclude that halving the oocyte cell size is helping in proper segregation of male meiosis I chromosomes in the cytoplasm of meiosis I oocytes. It is also possible that the experimental procedure involved in removing half of the cytoplasm is promoting proper segregation for some unknown reason. The authors should include a condition where half of the cytoplasm is aspirated but then put back again, so oocytes have the same volume as before but

the cytoplasm underwent the same treatment as in the halved oocytes. Also, increasing the cytoplasm volume of the oocyte should not lead to a better segregation of male chromosomes but make things worse, have the authors checked for that?
>> REPLY: We showed previously that the procedure of "aspirating half the cytoplasm and putting it back again" results in no detectable effects on meiosis in terms of the fidelity of chromosome segregation (Kyogoku & Kitajima, *Dev. Cell* 41: 287–298, 2017). Therefore, it is unlikely that the aspiration procedure *per se* affected the results. This result has been mentioned in the revised version (Lines 128–131). As for the experiment of increasing the cytoplasmic volume of oocytes, we showed that cytoplasmic enlargement increases chromosome segregation errors in oocytes (Kyogoku & Kitajima, *Dev. Cell* 41: 287–298, 2017). Because the experiment of cytoplasmic enlargement requires different procedures from those of cytoplasmic reduction (e.g., electrofusion), we are of the opinion that the experiment of cytoplasmic enlargement might not provide much useful information to the results of the present study.

2)The authors mention that male chromosomes align with a delay, compared to the female chromosomes. Does this delay depend on activation of error correction, or the spindle assembly checkpoint? Is it possible that dilution of factors required for checkpoint control and hence, assuring proper chromosome segregation, are the reason for error prone segregation in oocytes harboring twice the amount of chromosomes? If yes, have the authors stained for SAC proteins at the kinetochores? Maybe slight overexpression of the SAC protein were sufficient to rescue male meiotic divisions in the oocyte- have the authors tested this hypothesis?
>> REPLY: As the reviewer suggested, the possibility cannot be excluded that altered activity of the spindle assembly checkpoint (SAC) is involved in chromosome segregation errors in our model of biparental meiosis. To address this issue, we quantified the levels of SAC proteins (e.g., Mad2) at kinetochores in biparental meiosis. Consistent with the idea, spermatocyte-derived chromosomes appeared to fail the kinetochore recruitment of the SAC activator MAD2 (Figure 4C). However, halving ooplasmic volume did not recover MAD2 localization on spermatocyte-derived chromosomes (Figure 4C). Moreover, unlike halving ooplasmic volume, the forced activation of the SAC by tethering MAD1 at spermatocyte kinetochores with the MAD1-CENP-C fusion construct (Kyogoku and Kitajima 2017) did not efficiently rescue chromosome segregation errors in biparental meiosis (Figure 4D). Thus, although the SAC activation appears to be defective on spermatocyte chromosomes, it is likely that halving ooplasmic volume rescued chromosome segregation errors in biparental meiosis largely due to effects other than modifying the SAC activity (Lines 206–217).

3) The authors state that male chromosomes have a hard time segregating in the huge cytoplasm of the oocytes. Maybe it is not the fact that the chromosomes came from a male pronucleus, but this is just a manner of double the chromosomes that have to be segregated in the oocyte cytoplasm. How do male chromosomes behave in enucleated oocytes undergoing meiosis I? Conversely, if female chromosomes coming from another oocyte are injected into the recipient oocyte instead of male chromosomes, are those segregating correctly, or the delay in chromosome alignment and error rate comparable to the situation when the additional

chromosome set comes from the male?
>> REPLY: We agree that we should consider the possible influence of doubled chromosomal volume on the integrity of biparental meiosis. For the preparation of oocytes containing only the spermatocyte-derived chromosomes, we confirmed that we could enucleate the immature oocytes and inject a spermatocyte nucleus into them. However, most of the injected oocytes did not reach the MII stage. This might have been caused by the removal of meiosis-related factors from the oocytes at enucleation. So, unfortunately, we had to abandon the experiment with spermatocyte chromosome-only oocytes.

Another experimental setting—analysis of oocytes with doubled female chromosomes—was found to be technically feasible. When we transferred the MI stage-oocyte chromosomes into another MI oocyte to construct meiotic oocytes with doubled female chromosomes, they proceeded to the MII stage with a single chromosome mass and one first polar body. Our multicolor FISH analysis revealed that 9 (64%) out of 14 MII oocytes carried chromosomal abnormalities. These data suggest that the doubling of the number of chromosomes by nuclear/spindle transfer accounts for chromosome segregation errors irrespective of the origin of the donor nucleus/spindle. This is consistent with our findings that the lack of the SAC on the spermatocyte chromosome is not the major cause of their meiotic errors within recipient oocytes. These findings were incorporated into the revised version (Lines 218–228, Fig. S1).

4) In the rescue of mice with spermatogenic arrest the authors find aneuploidies of sex-chromosomes in the off-spring, not of autosomes. To my best of knowledge, autosome aneuploidies are not viable in the mouse, hence this result does not indicate that sex-chromosomes are the main source of aneuploidies. Nevertheless, it is attractive to speculate that aneuploidies are mainly due to sex chromosomes, because the oocyte is not prepared to segregate a male sex-chromosome bivalent. The authors should determine whether the segregation errors in meiosis I in oocytes harboring the additional male chromosome set concern mainly the male sex-chromosomes, by doing Fish analysis after meiosis I.
>> REPLY: We agree that this is an important point. To determine whether sex chromosomes are more vulnerable to biparental meiosis than the autosomes, we performed multicolor FISH using spermatocyte-injected MII oocytes. We found that both segregation errors occurred in both sex chromosomes and autosomes. This indicates that embryos with autosomal abnormalities most likely died before birth, as the reviewer pointed out. This result has been incorporated into the revised version (Lines 294–307, Fig. S5). Please also see the reply to Comment #2 from Reviewer 1.

(Significance (Required)):
This study is very interesting and of high significance, and very well executed. I think the study can go much further as far as mechanistic insights are concerned, only requiring techniques and tools that the authors have at their disposition.
>> REPLY: We appreciate the reviewer’s constructive suggestions very much. We hope that the reviewers will be satisfied with this revision that incorporated new cytochemical and cytogenetical analyses.

1st Editorial Decision **28th Mar 2022**

Dear Prof. Ogura,

Thank you for submitting your revised manuscript, which was previously reviewed at Review Commons. I have now heard back from all of the original referees. The referees find that the manuscript was significantly improved after revision and recommends publication in EMBO Reports. Before I can accept the manuscript, I need you to address some minor points below:

• As per our guidelines, please add a 'Data Availability Section', where you state that no data were deposited in a public database.
• We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Also, please rename the 'Conflict of Interests' section as 'Disclosure statement and competing interests'.
• As per our format requirements, in the reference list, citations should be listed in alphabetical order and then chronologically, with the authors' surnames and initials inverted; where there are more than 10 authors on a paper, 10 will be listed, followed by 'et al.'. Please see https://www.embopress.org/page/journal/14693178/authorguide#referencesformat
• Please fill out and include an author checklist as listed in out online guidelines (https://www.embopress.org/page/journal/14693178/authorguide)
• Please update the figure files as individual files.
• We note that the panels of Figures S3 & S5 are not called out in the text.
• We note that there is an Appendix file with 5 figures and 3 tables uploaded in one PDF file. The Appendix files need a Table of Contents and the nomenclature needs to be corrected as 'Appendix Figure S#' and 'Appendix Table S#'. The movie legends should be removed from the file. Alternatively, you can convert the content into Expanded View, in which case, the figures and the tables need to be uploaded as individual files and they need be renamed as 'Figure EV#' and 'Table EV#'.
• Please ZIP the movie files with their legends and rename them as 'Movie EV#'.
• Papers published in EMBO Reports include a 'synopsis' and 'bullet points' to further enhance discoverability. Both are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the paper (max 35 words) and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb and 3-5 bullet points listing the key experimental findings.
• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels. For example, you can adapt Figure 10 for this.
• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

Thank you again for giving us to consider your manuscript for EMBO Reports, I look forward to your minor revision.

Kind regards,

Deniz Senyilmaz Tiebe

--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

Referee #1:

The authors showed revised data and appropriate corrections in the manuscript, which overall sufficed my previous questions and concerns.

Referee #2:

The authors fully addressed my concerns and the manuscript is ready to proceed.

Referee #3:

The authors have addressed all the issues I raised in a satisfying manner. Therefore, I think the manuscript is now suitable for publication in Embo Reports.

_____

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Dr. Deniz Senyilmaz Tiebe, PhD.

Editor, EMBO Reports

Revision of a manuscript (EMBOR-2022-54992V1| [RC-2021-01059] [REV])

Dear Dr. Deniz Senyilmaz Tiebe,

Thank you and the reviewers very much for your careful and favorable evaluation of our revised manuscript (EMBOR-2022-54992V1). We are very pleased to know that the reviewers were satisfied with our revision and recommended publication in EMBO Reports, although we understand that the paper still needs minor revisions according to the journal's author guidelines, as you kindly instructed.

We carefully read your editorial instructions and have revised the manuscript. In addition to these revisions, we have shortened the title to fit the guideline (no more than 100 characters including the spaces). If you feel that the new title is not appropriate for EMBO Reports, please let me know.

The followings are the replies to your instructions. In the revised file, all changes to the text are indicated in red.

• As per our guidelines, please add a 'Data Availability Section', where you state that no data were deposited in a public database.

=> As suggested, we have added a 'Data Availability Section' and stated no data were deposited in a public database.

• We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Also, please rename the 'Conflict of Interests' section as 'Disclosure statement and competing interests'.

=> As suggested, we have put the ' Disclosure statement and competing interests' section and made statement "The authors declare that they have no competing interests."

• As per our format requirements, in the reference list, citations should be listed in alphabetical order and then chronologically, with the authors' surnames and initials inverted; where there are more than 10 authors on a paper, 10 will be listed, followed by 'et al.'. Please see https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

=> As suggested, we have modified the reference list.

• Please fill out and include an author checklist as listed in out online guidelines (https://www.embopress.org/page/journal/14693178/authorguide)

=> As suggested, we have filled the author checklist and uploaded.

• Please update the figure files as individual files.

=> We have uploaded high-resolution figures as individual files.

• We note that the panels of Figures S3 & S5 are not called out in the text.

=> We are sorry for this missing. Figures S3 and S5 are now Figures EV1 and EV3, respectively, and called out in the text (Lines 278 and 315).

• We note that there is an Appendix file with 5 figures and 3 tables uploaded in one PDF file. The Appendix files need a Table of Contents and the nomenclature needs to be corrected as 'Appendix Figure S#' and 'Appendix Table S#'. The movie legends should be removed from the file. Alternatively, you can convert the content into Expanded View, in which case, the figures and the tables need to be uploaded as individual files and they need be renamed as 'Figure EV#' and 'Table EV#'.

=> We understand the replacement of Supplemental information. We have moved three figures to Figures EV1 to EV3 (formerly Figure S3 to S5).

• Please ZIP the movie files with their legends and rename them as 'Movie EV#'.

=> As suggested, we have made ZIP files for four movies.

• Papers published in EMBO Reports include a 'synopsis' and 'bullet points' to further enhance discoverability. Both are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst summarizing the study in 1 or 2 sentences that summarize the paper (max 35 words) and are provided by the authors and streamlined by the handling editor. I would therefore ask you to include your synopsis blurb and 3-5 bullet points listing the key experimental findings.

=> As suggested, we have inserted 'synopsis' and 'bullet points' to the text.

• In addition, please provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels. For example, you can adapt Figure 10 for this.

=> As suggested, we have uploaded an image for the synopsis.

• Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated.

=> We have added necessary information to the figure legends. Please see the figure legends attached to the end of the main text file.

We greatly appreciate your kind instructions for the improvements of our manuscript. We hope you will be satisfied with this revision and will consider it suitable for publication in EMBO Reports.

Yours sincerely,

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ogura@rtc.riken.go.jp

1st Revision - Editorial Decision **19th Apr 2022**

Dear Prof. Ogura,

Thank you for submitting your revised manuscript. I have now looked at everything and all is fine. Therefore, I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice work!

Kind regards,

Deniz Senyilmaz Tiebe
--
Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

--
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Please inform us if there is likely to be any difficulty in reaching you at the above address at that time. Failure to meet our deadlines may result in a delay of publication, or publication without your corrections.

All further communications concerning your paper should quote reference number EMBOR-2022-54992V2 and be addressed to emboreports@wiley.com.

Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.

EMBO Press Author Checklist

Corresponding Author Name: Atsuo Ogura	
Journal Submitted to: EMBO Reports	
Manuscript Number: EMBOR-2022-54992V1	

USEFUL LINKS FOR COMPLETING THIS FORM

The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January 2022)

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	The sources (company and cat no.) of all the antibodies are described in the Materials and Methods. Only "rabbit anti-SGO2" is not commercially available, but can be obtained from the author.

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	In this study, genotyping of two mutant mouse lines was performed by PCR. The primer sequences for PCR are shown in the Materials and Methods

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	In a strict sense, we did not use any primary culture cells. But we freshly prepared spermatocytes for injection. All the information related to injected spermatocytes is shon in the Materials and Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	We used laboratory mice as models. All the I nformation related to mice used is shown in the Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	The housing and husbandry conditions of mice is also described in the Materials and Methods

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	All the necessary information on the sample size is shown in the Materials and Methods or Figure legends
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Statistical tests and their applications are shown in the Materials and Methods or Figure legneds, as appropriate.

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	All the necessary information (numbers of samples and replicates) is shown in figures or their legends, as appropriate
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	All the necessary information (numbers of samples and replicates) is shown in figures or their legends, as appropriate

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Information on the approval (with aproval numbers) of mouse experiments are shown in the Materials and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Yes	We state that all the mouse experiments were performed according to the principle of the ARRIVE guideline in the Materials and Methods
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	