Loss of ESRP1 Blocks Mouse Oocyte Development and Leads to Female Infertility

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DOI: 10.1242/dev.196931

Editor: Haruhiko Koseki

Review timeline
Original submission: 11 September 2020
Editorial decision: 23 October 2020
First revision received: 17 November 2020
Accepted: 24 November 2020

Original submission

First decision letter

MS ID#: DEVELOP/2020/196931

MS TITLE: ESRP1-Mediated Alternative Splicing During Oocyte Development is Required for Mouse Fertility

AUTHORS: Luping Yu, Huiru Zhang, Xuebing Guan, Dongdong Qin, Jian Zhou, and Xin Wu

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Epithelial splicing regulatory proteins (ESRPs) 1 and 2 participate in epithelial-mesenchymal crosstalk through the alternative splicing regulation. In this manuscript, the authors focused on oogenesis by using a germline-specific disrupted mouse line, and determined that ESRP1 is essential for spindle organization to enter into anaphase-I in the meiosis. Finally, the authors found the molecules responsible for causing inappropriate chromosome alignment by comprehensive screening. It is my opinion that this manuscript has the scientific novelty required for publication in Development.

Comments for the author

I think that this work gives a straightforward and accurate description of the main research subject. The only suggestion I have is that, because ESRP1 and its paralogue ESRP2 seem to be cooperative factors (Bebee et al., Elife 2015 PMID: 26371508), the authors should include some information regarding ESRP2 function in germline development.
Other than the above, in my opinion, the manuscript meets the criteria for publication in Development.

Reviewer 2

Advance summary and potential significance to field

In this manuscript the authors investigate the role of epithelial splicing regulatory protein 1 (ESRP1) in germ cells using conditional knockout mice. They find that germline deletion of Esrp1 gene leads to female infertility. ESRP1 conditional knockout mice show defects in follicular development and ovulation. They also find that the majority of ESRP1 deficient oocytes obtained after superovulation treatment fail to extrude the first polar body. Immunofluorescent analyses reveal that the loss of ESRP1 results in defects in meiotic spindle formation, leading to the activation of spindle assembly checkpoint and meiotic arrest. They further show that loss of ESRP1 in oocytes leads to changes in pre-mRNA splicing patterns of subset of genes including those involved in meiotic progression.
Overall, this study reports a novel role of ESRP1 in meiotic spindle formation with beautiful and convincing data and appears appropriate for Development. However, I also found several major and minor points that would need to be addressed before further consideration for publication.

Comments for the author

Major points

1) The authors clearly show that ESPR1 knockout mice show defects in oocyte maturation. They also show that loss of ESPR1 leads to alterations in splicing pattern of a set of maternal transcripts including those of oocyte meiosis-related genes. However, the causal relationship between the two are speculative and the title of this manuscript " ESRP1-Mediated Alternative Splicing During Oocyte Development is Required for Mouse Fertility" are not supported by the experimental data. From the results shown, authors cannot rule out the possibility that ESPR1 that is present in the cytoplasm (as evident in Figure 6) rather than in the nucleus, is involved in the spindle formation and chromosome segregation independently of the AS activity. Therefore authors should reconsider the title of the paper and the relevant section of the abstract (Lines 44-49), results (e.g. Lines 340-341, 377-378), and discussion (e.g. Lines 382-384, 437-439).

2) The data in Figure 4C should not be shown as a pie chart. This is because one oocyte may satisfy multiple phenotypes (Line 939), so adding all the values does not add up to 100.

3) While the data in Figures 5 and S4 are of high quality, there are several misstatements and overstatements in its description and interpretation.

— Page 10, Lines 262-263, “In contrast, the BubR1 signal on kinetochores was dramatically increased in Esrp1-knockout MI oocytes (Fig. 5A,B)”. The authors does not correctly describe the experimental data shown in Figures 5A and 5B. These data show that in Esrp1-knockout MI oocytes, chromosomes were not fully aligned even at the 6 hours after GVBD and the BubR1 signals at centromeres remained at prometaphase-I level.

— The authors claim that in Esrp1-knockout MI oocytes SAC is “abnormally” activated (e.g., Line 267 and 384), but do not provide data on which aspects are abnormal. The authors should either add an explanation for the abnormality by providing the data or rewrite it as "remains active".

— Page 10, Line 269, “We collected 10-h cultured oocytes from Esrp1 fl/fl and Esrp1 fl/fl /Gdf9-Cre females and… “ Does this mean that GV oocytes obtained from each females are cultured in vitro for 10 h and then collected for the analysis?

— Page 10, Lines 273-274. “both proteins were present during the metaphase-to-anaphase transition…”. The phrase “during metaphase-to-anaphase transition” should not be used for the Esrp1-knockout oocytes because the majority of them fails to enter anaphase I, but should be replaced with, for example, “even after 10 h-culture”.

— In Figure S4A-D, authors nicely demonstrate that Reversin treatment dramatically increase the ratio of successful PBE among Esrp1-knockout oocytes, but still only about 50%. Therefore, to reach the conclusion that inhibition of SAC activity rescued metaphase-I arrest caused by Esrp1 deficiency (Lines 290-291), it is important to show that even in the oocytes that failed to extrude the 1st polar body after Reversin treatment, bivalent chromosomes are rarely observed, or to show that the cyclin B and securin are decreased to undetectable level after reversing treatment.

Minor points

1) Page 9, Lines 231-234. “On meiotic entry, dynamic microtubules form a bipolar spindle, which is responsible for capturing and congressing chromosomes. These events require proper attachment of kinetochores to microtubules emanating from opposite spindle poles (Touati et al., 2015).” I think this is misleading because a previous paper demonstrate that in mouse oocyte meiosis I, chromosome congression precedes establishment of bioriented Kt-mictorutube atattchment (Kitajima et al., Cell 146, 568-581, 2011).

2) Why do the authors use the term “pre-metaphase I” (e.g. line 259) ?. In Figure 5 the term “prometaphase I” is used and does not match; I suggest prometaphae I is preferable.

Reviewer 3

Advance summary and potential significance to field

Alternative splicing (AS) of pre-mRNA contributes to gene diversification in cells, but the importance of AS during germline development remains largely undefined. This report provide evidence that an epithelial splicing regulatory protein, ESRP1, has critical roles for spindle organization, chromosome segregation, and follicular development in female germline. These findings indicates indispensable involvements in AS of female oogenesis and their fertility.

Comments for the author

This study reveals the importance of AS in oogenesis by oocyte-specific KO of one of the RBPs, ESRP1. Authors showed that AS insufficiency causes spindle formation and chromosome segregation, and metaphase-to anaphase transformation in oocytes. Although some important results were shown in the manuscript, some crucial results or details also should be provided to improve the quality before the publication.

Major concerns
1. The phenotype of female Esrp1fl/fl/Ddx4-cre is inadequately described by infertility alone. Ovarian sections in newborn pups and sexually mature individuals need to be analyzed.

2. Fig. 5c. Confirm whether APC/C is present but not functioning or reduced.

Minor concerns
1. FIg.1E. Indicate the time point when the ovaries of Esrp1fl/fl/Gdf9-cre were prepared?
2. line 422. Indicate "few studies" as refs.
3. line 643. 1-2 min exposure of Tyrode's solution, oocytes must be degenerating.
4. line 933. Not arrows, but arrowheads.
5. Fig. 6B. Not GN-SN, but GV-SN.
6. Fig. S3C. Zygote means that oocytes were fertilized with sperm. Indicate the evidence for these oocytes surely fertilized with sperm.

First revision

Author response to reviewers' comments

We thank the editor for his consideration on our manuscript. We also thank all reviewers for their valuable comments regarding our work which help us to improve the quality of our manuscript. We are pleased to submit a revision by addressing each comment with either additional experiments or revision of the text. Based on comments from reviewer 2, we revised the title to "Loss of ESRP1 Blocks Mouse Oocyte Development and Leads to Female Infertility". The reversion is submitted with all changes highlighted in red.

Collectively, we hope all the comments and concerns have been sufficiently addressed in this revision, and sincerely thank the editor for the time and consideration of this manuscript for the publication in *Development*.

Our point-by-point responses to each comment are as follows:

Reviewer 1:

Comments for the author:
1. I think that this work gives a straightforward and accurate description of the main research subject. The only suggestion I have is that, because ESRP1 and its paralogue ESRP2 seem to be cooperative factors (Bebee et al., Elife 2015 PMID: 26371508), the authors should include some information regarding ESRP2 function in germline development. Other than the above, in my opinion, the manuscript meets the criteria for publication in Development.

Response: We appreciate all aspects of these comments. We fully agree with the reviewer that ESRP2 has cooperative roles with ESRP1 in the cleft lip and epithelial cell functions according to the suggested paper, which has been cited in our manuscript as well(*Bebee et al., Elife 2015 PMID: 26371508*). Before we started this project, we had carefully checked the expression of ESRP2 in mouse germline; however, neither RBP pull-down experiments nor previous mRNA profiling data supported the expression of ESRP2 in germline cells. We have attached our real-time RT-PCR and RNA single-cell sequencing data in mouse oocytes for the reviewer to check (attached below). In response to this valid comment, we have added several additional sentences in our result parts (page 5, line 126-130) to clarify that the expression of ESRP2 is likely absent in mouse germline.

1) Real-time RT-PCR analysis of mRNA levels of *Esrp1* and *Esrp2* in GV-stage and MII- stage oocytes from *Esrp1*$^{fl/fl}$ mice. (n=3, *p < 0.05).

We have removed unpublished data provided for the referees in confidence.

2) RNA single-cell sequencing Data (RNA-seq; four biological replicates in each group, GEO database GSE149355).

We have removed unpublished data provided for the referees in confidence.

Reviewer 2:

Comments for the author:

Major points:
1. The authors clearly show that ESPR1 knockout mice show defects in oocyte maturation. They also show that loss of ESPR1 leads to alterations in splicing pattern of a set of maternal transcripts including those of oocyte meiosis-related genes. However, the causal relationship between the two are speculative and the title of this manuscript "ESRP1-Mediated Alternative Splicing During Oocyte Development is Required for Mouse Fertility" are not supported by the experimental data. From the results shown, authors cannot rule out the possibility that ESPR1 that is present in the cytoplasm (as evident in Figure 6) rather than in the nucleus, is involved in the spindle formation and chromosome segregation independently of the AS activity. Therefore, authors should reconsider the title of the paper and the relevant section of the abstract (Lines 44-49), results (e.g. Lines 340-341, 377-378), and discussion (e.g. Lines 382-384, 437-439).

Response: We appreciate the comments from the reviewer. We agree that ESPR1 is also present in oocyte cytoplasm. Therefore, we cannot exclude the possibility that the defects of spindle formation and chromosome segregation are also due to the loss function of ESPR1 in cytoplasm. We have revised the title to "Loss of ESRP1 blocks mouse oocyte development and leads to female infertility". We have weakened our claims and made few modifications in the abstract (page 2, line 44; page 2, line 46) and results (page 13, line 355-356; page 14, line 393). We also revised our words in discussion section (page 17, line 454-458) in the revised manuscript, accordingly.

2. The data in Figure 4C should not be shown as a pie chart. This is because one oocyte may satisfy multiple phenotypes (Line 939), so adding all the values does not add up to 100.

Response: We agree with your comment. We have revised this figure to a column chart (Fig. 4C).

3. While the data in Figures 5 and S4 are of high quality, there are several misstatements and overstatements in its description and interpretation.

—**Page 10, Lines 262-263**,"In contrast, the BubR1 signal on kinetochores was dramatically increased in Esrp1-knockout MI oocytes (Fig. 5A,B). The authors does not correctly describe the experimental data shown in Figures 5A and 5B. These data show that in Esrp1-knockout MI oocytes, chromosomes were not fully aligned even at the 6 hours after GVBD and the BubR1 signals at centromeres remained at prometaphase-I level.

Response: According to the suggestion, we have revised the description to "in *Esrp1*- knockout MI oocytes, chromosomes were not fully aligned even at the 6 hours after GVBD and the BubR1 signals at centromeres remained at pro-MI level" (page 10, line 274-276).

—**The authors claim** that in Esrp1-knockout MI oocytes SAC is "abnormally" activated (e.g., Line 267 and 384), but do not provide data on which aspects are abnormal. The authors should either add an explanation for the abnormality by providing the data or rewrite it as "remains active".

Response: According to the suggestion, we have revised the description to "continuously active SAC" (page 15, line 400-401).

—**Page 10, Line 269**, "We collected 10-h cultured oocytes from *Esrp1*$^{fl/fl}$ and *Esrp1*$^{fl/fl}$/*Gdf9-Cre* females and… " Does this mean that GV oocytes obtained from each females are cultured in vitro for 10 h and then collected for the analysis?

Response: Yes, the GV oocytes obtained from females are cultured *in vitro* for 10 h and then collected for the analysis. According to the suggestion, we have revised the description to "GV

oocytes from *Esrp1*$^{fl/fl}$ and *Esrp1*$^{fl/fl}$/*Gdf9-Cre* females, cultured *in vitro* for 10 h" (page 10, line 282-283).

—Page 10, Lines 273-274. "both proteins were present during the metaphase-to-anaphase transition…". The phrase "during metaphase-to-anaphase transition" should not be used for the Esrp1-knockout oocytes because the majority of them fails to enter anaphase I, but should be replaced with, for example, "even after 10 h-culture".

Response: Thank you for the suggestion. We revised the description to "the presences of cyclin B1 and securin after 10 h-culture in *Esrp1*- knockout oocytes" (page 11, line 286-287).

—In Figure S4A-D, authors nicely demonstrate that Reversin treatment dramatically increase the ratio of successful PBE among Esrp1-knockout oocytes, but still only about 50%. Therefore, to reach the conclusion that inhibition of SAC activity rescued metaphase-I arrest caused by Esrp1 deficiency (Lines 290-291), it is important to show that even in the oocytes that failed to extrude the 1st polar body after Reversin treatment, bivalent chromosomes are rarely observed, or to show that the cyclin B and securin are decreased to undetectable level after reversing treatment.

Response: It has been shown that Reversin treatment could rescue the metaphase arrest (J Cell Biol. 2019 PMID: 30723090). In response to this valid comment, we used chromosome-spread assay and showed that the proportion of homologous chromosomes separation and disappearance of bivalent chromosomes were approximately 73% among the oocytes that failed to extrude the 1st polar body, indicating the Reversin treatment could partially rescue the metaphase arrest in *Esrp1* deficient mice. These data and information have been provided at Fig S5G and page 11, line 301-304.

Minor points:
1. Page 9, Lines 231-234. "On meiotic entry, dynamic microtubules form a bipolar spindle, which is responsible for capturing and congressing chromosomes. These events require proper attachment of kinetochores to microtubules emanating from opposite spindle poles (Touati et al., 2015)." I think this is misleading because a previous paper demonstrate that in mouse oocyte meiosis I, chromosome congression precedes establishment of bioriented Kt-mictorutube atattchment (Kitajima et al., Cell 146, 568-581, 2011).

Response: We appreciate the reviewer to point out this conceptual mistake. According to the suggestion, we revised the description to "On meiotic entry, chromosome congression forms an intermediate chromosome configuration, and invades the elongating spindle center to form the metaphase plate before biorienting. Close to 90% of all chromosomes will have one or more rounds of error correction of their kinetochore-microtubule (K-MT) attachments and eventually generate correct biorientation" and used the reference suggested by the reviewer (Kitajima et al., Cell 146, 568-581, 2011, PMID: 21854982) (page 9, line 241-245).

2. Why do the authors use the term "pre-metaphase I" (e.g. line 259)? In Figure 5 the term "prometaphase I" is used and does not match; I suggest prometaphase I is preferable

Response: Thank you for the suggestion. We have revised the description to "prometaphase I" through the whole text (page 10, line 271; 34, line 994, line 998).

Reviewer 3:

Comments for the author:

Major points:
1. The phenotype of female Esrp1fl/fl/Ddx4-cre is inadequately described by infertility alone. Ovarian sections in newborn pups and sexually mature individuals need to be analyzed.

Response: Thank you for the suggestion. We have added the morphology of six-week-old ovaries from *Esrp1*$^{fl/fl}$ and *Esrp1*$^{fl/\Delta}$/*Ddx4-Cre* mice in Fig. 1C and immunofluorescent staining data of ESRP1

(red) in PD21 mouse ovaries from *Esrp1*$^{fl/\Delta}$/*Ddx4-Cre* in Fig. 1G. HE staining of six-week-old *Esrp1*$^{fl/fl}$ and *Esrp1*$^{fl/\Delta}$/*Ddx4-Cre* ovaries sections (Fig. S1C) indicated there are various stages of developing oocytes in the ovary, which exclude the possibility of completely loss of germ cells before birth. Therefore, we did not check the newborn pups. The authors might check for further experimental purposes, e.g. to investigate whether germ cell pool will reduce at birth in *Esrp1*$^{fl/\Delta}$/*Ddx4-Cre* ovary. The data and information have been provided at Figs. 1C, 1G and S1C and page 5-6, line 143-148.

2. Fig. 5c. Confirm whether APC/C is present but not functioning or reduced.

Response: In response to this valid comment, we have generated additional experiments to verify the present and functioning of APC/C (anaphase-promoting complex/cyclosome, a 1.2-MDa ubiquitin ligase complex). We evaluated the protein levels of APC2 (an subunit of APC/C), (Science, 1998 PMID: 9469814), CDC20 (an essential substrate adaptor of APC/C), (PLoS Genet, 2010 PMID: 20941357). As the results, we concluded that APC/C is still present but functioning is reduced in the oocytes of *Esrp1*$^{fl/fl}$/*Gdf9-cre* mice. These data and information have been provided at Fig. S4 and page 10-11, line 282-289.

Minor concerns
1. FIg.1E. Indicate the time point when the ovaries of Esrp1fl/fl/Gdf9-cre prepared?
Response: Thank you for this suggestion, we removed this data since we have provided the immunofluorescence data.

2. line 422. Indicate "few studies" as refs.
Response: The references have been provided (Do et al., 2018 PMID: 29928511; Kasowitz et al., 2018 PMID: 29799838) (page 16, line 439).

3. line 643. 1-2 min exposure of Tyrode's solution, oocytes must be degenerating.
Response: Thank you for your suggestion, we have corrected the words as “most oocytes need 10-20s to finish this process” (page 24, line 665-666).

4. line 933. Not arrows, but arrowheads.
Response: Thank you to point out this error. We have corrected the words through the text (page 8, line 201; page 32, line 948; page 33, line 955; page 33, line 973).

5. Fig. 6B. Not GN-SN, but GV-SN.
Response: The typo has been corrected in Fig. 6B.

6. Fig. S3C. Zygote means that oocytes were fertilized with sperm. Indicate the evidence for these oocytes surely fertilized with sperm.
Response: Thank you for the comment. We have removed the unnecessary information in the Fig. S3C and corresponding text (page 8, line 214-216).

Second decision letter

MS ID#: DEVELOP/2020/196931

MS TITLE: Loss of ESRP1 Blocks Mouse Oocyte Development and Leads to Female Infertility

AUTHORS: Luping Yu, Huiru Zhang, Xuebing Guan, Dongdong Qin, Jian Zhou, and Xin Wu
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

In second round of review, I think that the authors have improved all concerns appropriately.

Comments for the author

I am satisfied with the modifications provided by the authors.

Reviewer 2

Advance summary and potential significance to field

The authors have revised the manuscript by answering all comments and questions from reviewers, showing the additional data needed.

Comments for the author

I have no further comments and I think the revised manuscript is suitable for publication.

Reviewer 3

Advance summary and potential significance to field

This study reveals the importance of AS in oogenesis by oocyte-specific KO of one of the RBPs, ESRP1. Authors showed that AS insufficiency causes spindle formation and chromosome segregation, and metaphase-to anaphase transformation in oocytes. Revised version of this manuscript will provide novel knowledge that AS has critical involvement of female oogenesis and their fertility.

Comments for the author

I think no other revision is needed for publication.