EMBO Molecular Medicine

Bi-allelic Pathogenic Variants in PABPC1L Cause Oocyte Maturation Arrest and Female Infertility

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8th Dec 2022

Dear Dr. Wang,

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now received feedback from the three reviewers who agreed to evaluate your manuscript. As you will see from the reports, all three referees support publication of the study but they also raise important concerns that should be addressed in a major revision.

Further consideration of a revision that addresses reviewers' concerns in full will entail a second round of review. EMBO Molecular Medicine encourages a single round of revision only and therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. For this reason, and to save you from any frustrations in the end, I would strongly advise against returning an incomplete revision.

We would welcome the submission of a revised version within three months for further consideration. Please let us know if you require longer to complete the revision.

I look forward to receiving your revised manuscript.

Yours sincerely,

Zeljko Durdevic

Zeljko Durdevic
Editor
EMBO Molecular Medicine

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When submitting your revised manuscript, please carefully review the instructions that follow below. We perform an initial quality control of all revised manuscripts before re-review; failure to include requested items will delay the evaluation of your revision.

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1) A .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

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https://www.embopress.org/page/journal/17574684/authorguide#dataavailability).

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10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

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***** Reviewer's comments *****

Referee #1 (Remarks for Author):

In the work of Weijie Wang et al, exome studies of five affected patients from four families exhibiting infertility with maturation arrest and unsuccessful IVF or ICSI were performed. They allowed to identify compound heterozygous or homozygous pathogenic or likely pathogenic coding missense or truncating variants of PABPC1L, the predominant polyA binding protein in human oocytes and early embryos, and one missense variant in the 3' non coding region. These variants are functional in vitro since they yield truncating proteins, lower expression of the protein or induced mislocalization in HELA cells except for the p.Arg374Gln variant. All the missense variants except the p.Arg374Gln variant led to a significant decrease in mRNA translational activity in Hela cells. RNA pull-down experiments revealed that all missense variants except the p.Arg374Gln variant dramatically reduced the RNA-binding ability of the WT PABC1L. Knock in mice corresponding to the G97D and the S137F variants were generated by the CRISPR-Cas 9 technology. These mice ovulated mature oocytes, the fertilization rates of KI oocytes was normal however zygotes arrested at an early stage and failed to develop into blastocysts at 96h after fertilization. The variants are thus pathogenic with species-specific differences in humans and mice. Of note in family 2 and 3 however few retrieved oocytes could be fertilized but embryos obtained arrested at an early stage. RNA-seq studies showed that the upstream activator of the MAPK, Mos, was significantly increased in G97D and S137F zygotes. Qt-PCR confirmed this higher expression. Significantly elevated pERK1/2 levels were found in G97D and S137F zygotes strongly suggesting an activation of the Mos-MAPK pathway in Pabpc1l Ki zygotes. Micro injection of human MOS cRNAs indicates that the PABPC1L pathogenic variants lead to an increased Mos expression and activation of the Mos-MAPK pathway in zygotes, resulting in early embryonic arrest and female infertility in knock-in mice.

This work is interesting. Experiments and results are clearly described This is a comprehensive study with in vitro and in vivo knock in experiments. A novel mechanism of oocyte maturation arrest in humans is reported in this study.
Several points need to be clarified :
In the introduction and the discussion causes of oocyte maturation arrest in humans are reported. ECAT1 and ZP1,2,3,4 should also be discussed.
In Family 3 there is only one child. Is there any explanation? How old was the mother at the time of pregnancy? Has she had any failed attempts to get pregnant? This would argue for a functional effect of a heterozygous variant in humans.
No significant in vitro or in vivo functional effect is described for the p.Arg374Gln variant, homozygous in family 4. A short list of variants from exome analysis could be discussed to ensure that no other causative gene could be involved in the infertility of this family. Without functional effect or any other cause identified, no causal evidence is provided for family 4. It should be removed from this study.

Referee #2 (Comments on Novelty/Model System for Author):

Original findings. Proof of the functionality/causality of the newly identified mutations

Referee #2 (Remarks for Author):

This study identified for the first time pathogenic variants in the PABPC1L gene, in five patients suffering from mainly oocyte maturation arrest. Thorough functional analysis of these variants was performed in vitro and in mouse to confirm their causality, which makes the paper scientifically sound. In addition, the authors aimed to unravel the molecular cascade leading to the observed phenotype, by performing RNAseq analysis and assessment of the Mos-MAPK pathway. Assessment of this pathway feels a bit arbitrarily chosen, since it was not the most prominent DEG, and it is a pity that the RNAseq data was not assessed in a more unbiased manner. The authors should address this.

Concerning the results section, a lot of the text can be found back in the tables and figures, so some reduction of text should be performed. For example, table 1 contains all the details of patients which are easy to follow, so it's not necessary to add all the descriptive traits of involved patients in the results section.

Regarding the KI experiments in mice, how was the gene editing efficiency confirmed in the KI mice? For example, was there any mosaic gene editing observed which could bias the infertility phenotype? The authors performed IVF in the generated KI mice. Was in vivo reproduction successful in these KI mice? Are the authors capable of performing ICSI? Sometimes after performing ICSI, a more severe infertility phenotype can be observed compared to IVF, for example with PLCz KO male mice.

Another point to address is the lack of experimental analysis on the oocytes from these patients during/after infertility treatment. The authors should acknowledge this shortcoming.

Finally, it would be of added value to know from the authors which possible treatments options could be investigated to overcome this infertility problem? Nonetheless, the manuscript is clearly organized and well written. The study is of high quality and therefore, I support these results suitable for publication EMBO Molecular Medicine, following some minor modifications according to the comments listed below.

Minor remarks
- Line 28: I would not describe it as a common cause
- Line 115: PB1 oocyte is not a commonly used term, compared to GV and MI/MII oocytes, so please revise this
- Line 145: "The affected individual in family 2 carried the compound heterozygous variants consisting of a missense variant c.410C>T (p.Ser137Phe) and a frameshift insertion variant c.956_957insA (p.Ile320Asnfs*122)."
  The sentence might need some restructuring. An individual carrying the heterozygous variants consisting of ... feels as incorrect use of terminology.
- Line 164: "We measured the expression of PABPC1L mRNA and found that PABPC1L was highly expressed in human immature GV and MI oocytes as well as in the lung, but was poorly expressed in mature MII oocyte, early embryos, and other somatic tissues (Fig 1D), suggesting an important role for PABPC1L in human oocyte maturation."
  Clarify in the manuscript that this data was obtained in control samples.
- Line 170: "To evaluate the functional effects of the identified pathogenic variants in vitro, we first performed immunoblot analysis in HeLa cells transfected with WT or mutant PABPC1L constructs."
  Clarify in the manuscript what type of constructs: plasmid delivery
- Line 248: Here, I would first describe the data more generalized and only towards the end of the paragraph start focussing on the Mos-MAPK, to show that you work from generalized interpretation to targeted interpretation. Clarify a bit more why you focus on the Mos-MAPK in particular, and not the other highly differentially expressed genes.
- Line 297: "The infertile phenotype can be explained by abnormal activation of the Mos-MAPK pathway in zygotes."
  I believe this statement is too strong. For sure, Mos-MAPK is dysregulated in this model, but this is only one step in the molecular cascade leading to the observe phenotype. Please reconsider this statement.
  In the discussion, you approach this more carefully, which is better.
- Line 337: "Furthermore, all embryos obtained from the affected individuals in families 2 and 3 were arrested at an early stage, suggesting that PABPC1L also play an important role in early embryonic development prior to ZGA."
  I would rather state that zygote cleavage is compromised, given the drop in developmental rate between zygote and 2-cell stage shown in figure 4B.
- Line 368: "In addition, Pabpc1l KI mice harbouring homozygous missense variants had a milder phenotype than affected individuals in families 1 and 2 harboring compound heterozygous variants consisting of a missense variant and a truncating variant."
  I would remove this sentence and keep comparisons between different genotypes within one species. So I would only compare mouse with mouse (so KO mouse with KI mouse) and human with human (missense with nonsense).
- Discussion: You identified novel pathogenic variants in PABPC1L leading to the observed phenotype, but as mentioned in the introduction, the genetic basis and the mechanisms involved in the majority of affected individuals remain unknown. To the discussion, you could add the frequency/pick-up rate of variants in the PABPC1L gene and add some speculation on the missing genetic basis in the majority of patients. In what type of pathways could we still find novel pathogenic variants?
- Line 541: "Clean reads were aligned to the human genome hg38 using STAR and StringTie."
  wasn't RNAseq performed on mouse?

- Line 558: The journal doesn't require you to deposit the raw sequencing data in a database?

Referee #3 (Remarks for Author):

This interesting and well-written manuscript by Wang et al identifies several infertile woman who exhibit defective oocyte development, and find that they bear biallelic mutations in the very interesting gene PABPC1L. In most cases, pedigree genotyping was possible, enabling the authors to reveal phasing and a recessive mode of inheritance. This paper is distinguished by the validation experiments performed by the authors. They performed various assays tailored to the allele type (e.g., mismatches vs premature stops), including transfection of mutant constructs into HeLa cells to confirm predicted protein truncations and functional defects in reporter gene translation or RNA binding. Critically, the authors generate mouse models for the nonsynonymouse alleles, and find that they cause infertility phenotypes that essentially confirm the pathogenicity of the human alleles (and reveal subtle differences in mouse vs human phenotypes that are probably reflective of species differences).

Overall, this is an elegant paper with no significant weaknesses. The data support the conclusions. The experimental confirmations of variant effects set this paper aside from many other reports of infertility-causing alleles with far less evidence. I make only the following 3 minor suggestions.

- last sentence of abstract. It isn't clear if the "new marker for genetic diagnosis..." is PABPC1L or MOS mRNA levels. PABPC1L isn't really a "marker" but potential candidate to be screened for causes of infertility.

- Results section starts abruptly on line 104. Maybe an introductory sentence about the patient pool.

- sequence traces probably unnecessary in Fig. 1 (could go to Supplemental).

Responses to reviewers

***** Reviewer's comments *****

Referee #1 (Remarks for Author):

In the work of Weijie Wang et al, exome studies of five affected patients from four families exhibiting infertility with maturation arrest and unsuccessful IVF or ICSI were performed. They allowed to identify compound heterozygous or homozygous pathogenic or likely pathogenic coding missense or truncating variants of PABPC1L, the predominant polyA binding protein in human oocytes and early embryos, and one missense variant in the 3' non coding region. These variants are functional in vitro since they yield truncating proteins, lower expression of the protein or induced mislocalization in HELA cells except for the p.Arg374Gln variant. All the missense variants except the p.Arg374Gln variant led to a significant decrease in mRNA translational activity in Hela cells. RNA pull-down experiments revealed that all missense variants except the p.Arg374Gln variant dramatically reduced the RNA-binding ability of the WT PABC1L. Knock in mice corresponding to the G97D and the S137F variants were generated by the CRISPR-Cas 9 technology. These mice ovulated mature oocytes, the fertilization rates of KI oocytes was normal however zygotes arrested at an early stage and failed to develop into blastocysts at 96h after fertilization. The variants are thus pathogenic with species-specific differences in humans and mice. Of note in family 2 and 3 however few retrieved oocytes could be fertilized but embryos obtained arrested at an early stage. RNA-seq studies showed that the upstream activator of the MAPK, Mos, was significantly increased in G97D and S137F zygotes. Qt-PCR confirmed this higher expression. Significantly elevated pERK1/2 levels were found in G97D and S137F zygotes strongly suggesting an activation of the Mos-MAPK pathway in Pabpc1l Ki zygotes. Micro injection of human MOS cRNAs indicates that the PABPC1L pathogenic variants lead to an increased Mos expression and activation of the Mos-MAPK pathway in zygotes, resulting in early embryonic arrest and female infertility in knock-in mice.This work is interesting. Experiments and results are clearly described This is a comprehensive study with in vitro and in vivo knock in experiments. A novel mechanism of oocyte maturation arrest in humans is reported in this study.
Response: Thank you for your positive assessment.

Several points need to be clarified :
In the introduction and the discussion causes of oocyte maturation arrest in humans are reported. ECAT1 and ZP1,2,3,4 should also be discussed.
Response: According to your suggestion, we have discussed the role of *ZP1,2,3,4* in human oocyte maturation as following: “Zona pellucida is an extracellular glycoprotein matrix composed of ZP1, ZP2, ZP3, and ZP4, which plays a vital role in oocyte maturation. Studies have reported that the variants in *ZP1*(MIM: 195000), *ZP2* (MIM: 182888), and *ZP3* (MIM: 182889) affect the formation of zona pellucida and result in female infertility (Dai *et al*, 2019; Huang *et al*, 2014; Liu *et al*, 2017), but there is no convincing evidence to prove that *ZP4* (MIM: 613514) variant responsible for female infertility”. We have added this into the Introduction section (Line 65-71).

Reference:
1. Dai C, Hu L, Gong F, Tan Y, Cai S, Zhang S, Dai J, Lu C, Chen J, Chen Y et al (2019) ZP2 pathogenic

variants cause in vitro fertilization failure and female infertility. *Genet Med* 21: 431-440
2. Huang HL, Lv C, Zhao YC, Li W, He XM, Li P, Sha AG, Tian X, Papasian CJ, Deng HW et al (2014) Mutant ZP1 in familial infertility. *N Engl J Med* 370: 1220-1226
3. Liu W, Li K, Bai D, Yin J, Tang Y, Chi F, Zhang L, Wang Y, Pan J, Liang S et al (2017) Dosage effects of ZP2 and ZP3 heterozygous mutations cause human infertility. *Hum Genet* 136: 975-985

In Family 3 there is only one child. Is there any explanation? How old was the mother at the time of pregnancy? Has she had any failed attempts to get pregnant? This would argue for a functional effect of a heterozygous variant in humans.
Response: In family 3, there is only one child. The mother was pregnant at the age of 18 and gave birth to her daughter (Individual II-1) successfully. The mother didn't have any failed attempts to get pregnant.

No significant in vitro or in vivo functional effect is described for the p.Arg374Gln variant, homozygous in family 4. A short list of variants from exome analysis could be discussed to ensure that no other causative gene could be involved in the infertility of this family. Without functional effect or any other cause identified, no causal evidence is provided for family 4. It should be removed from this study.
Response: Thanks for the suggestion. To further confirm the pathogenicity of the variant c.1121G>A (p.Arg374Gln), we previously constructed *Pabpc1l* R374Q KI mice corresponding to this variant. During the reviewing of the manuscript, the KI mice has been produced. Phenotypic evaluation found that *Pabpc1l*$^{R374Q/R374Q}$ female mice were infertile characterized by early embryonic arrest (Fig 4A–C), which was consistent with the other two KI mice. In addition, immunofluorescence analysis showed that the level of pERK1/2 in the R374Q KI zygotes was significantly increased (Fig 6C), indicating that the Mos-MAPK pathway was also abnormally activated. These results fully demonstrate that the variant c.1121G>A (p.Arg374Gln) identified in family 4 has disruptive effects on protein function and ultimately leads to female infertility. We inferred that the biochemical mechanism of Arg374Gln *in vitro* may be different from other variants, but all variants have the similar pathological mechanism *in vivo*. We have added the relevant description to the Discussion section (Line 357-368).

Referee #2 (Comments on Novelty/Model System for Author):
Original findings. Proof of the functionality/causality of the newly identified mutations

Referee #2 (Remarks for Author):

This study identified for the first time pathogenic variants in the PABPC1L gene, in five patients suffering from mainly oocyte maturation arrest. Thorough functional analysis of these variants was performed in vitro and in mouse to confirm their causality, which makes the paper scientifically sound. In addition, the authors aimed to unravel the molecular cascade leading to the observed phenotype, by performing RNAseq analysis and assessment of the Mos-MAPK pathway. Assessment of this pathway feels a bit arbitrarily chosen, since it was not the most prominent DEG, and it is a pity that the RNAseq data was not assessed in a more unbiased manner. The authors should address this.
Response: Thank you for the suggestion. At the end of the analysis of RNA-seq data, we explain the reason why we chose to assess Mos-MAPK pathway as following “To further investigate the molecular mechanism,

we first analyzed the differentially expressed genes in G97D zygotes and mainly focused on oocyte-specific genes. We found that *Mos*, an upstream activator of MAPK cascade (Dupre *et al*, 2011), was the only oocyte-specific gene among the top 30 differentially expressed genes (Dataset EV1, Fig 5B). Then we checked the expression of *Mos* in S137F zygotes and observed that it was also up-regulated (Dataset EV1, Fig 5B). The up-regulation of *Mos* in KI zygotes is consistent with the activation of MAPK cascade revealed in GO analysis. The above RNA-seq analysis results strongly suggest that Mos-MAPK pathway is abnormally activated in KI zygote. In addition, studies have indicated that abnormal activation of Mos-MAPK pathway in embryo led to cleavage arrest (Haccard *et al*, 1993; Sagata *et al*, 1989; Verlhac *et al*, 2000), which is similar to the phenotype of early embryonic arrest in KI mice. Therefore, we supposed that the abnormal up-regulation of *Mos* expression and activation of the Mos-MAPK pathway in KI zygotes might be the potential mechanism of early embryonic arrest in *Pabpc1l* KI mice". We have added this into the Result section (Line 252-266).

Concerning the results section, a lot of the text can be found back in the tables and figures, so some reduction of text should be performed. For example, table 1 contains all the details of patients which are easy to follow, so it's not necessary to add all the descriptive traits of involved patients in the results section.
Response: According to your suggestion, we have removed some redundant descriptive traits of involved patients in the Results section.

Regarding the KI experiments in mice, how was the gene editing efficiency confirmed in the KI mice? For example, was there any mosaic gene editing observed which could bias the infertility phenotype?
Response: In this study, the KI mice we used are F2 generation mice, and they can stably inherit the mutation to their offspring. In the mouse phenotype evaluation, all homozygous KI mice showed the same phenotype, and no difference was observed in these mice.

The authors performed IVF in the generated KI mice. Was in vivo reproduction successful in these KI mice? Are the authors capable of performing ICSI? Sometimes after performing ICSI, a more severe infertility phenotype can be observed compared to IVF, for example with PLCz KO male mice.
Response: *Pabpc1l* KI female mice constructed in this study failed to reproduce *in vivo* and showed complete infertility (Fig 4A). In addition, according to your suggestion, we used G97D KI oocytes for ICSI experiments and found that KI oocytes can normally fertilize and form pronucleus after ICSI. However, in contrast to the WT zygotes, KI zygotes eventually arrested at an early stage and failed to develop into blastocysts, which is consistent with the results of IVF (Fig R1).

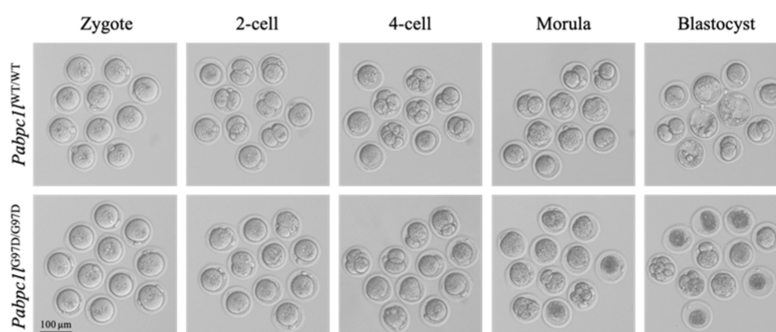

Figure R1 The early embryonic development at different times after ICSI of superovulated oocytes from WT and *Pabpc1l* G97D KI mice.

Another point to address is the lack of experimental analysis on the oocytes from these patients during/after infertility treatment. The authors should acknowledge this shortcoming.

Response: In this study, because we failed to obtain valuable clinical samples from these patients, we did not conduct experimental analysis on the oocytes from these patients, which is indeed a shortcoming of this study. We have pointed out this shortcoming in the Discussion section (Line 402-404).

Finally, it would be of added value to know from the authors which possible treatments options could be investigated to overcome this infertility problem? Nonetheless, the manuscript is clearly organized and well written. The study is of high quality and therefore, I support these results suitable for publication EMBO Molecular Medicine, following some minor modifications according to the comments listed below.

Response: Thanks for your constructive comments and appreciation on this work. Previous studies have found that microinjection of *Pabpc1l* mRNA into *Pabpc1l*$^{-/-}$ preantral follicle-enclosed oocytes rather than denuded GV oocytes could rescue oocyte maturation (Guzeloglu-Kayisli *et al.*, 2012; Lowther & Mehlmann, 2015). This strategy might provide a possible treatment option to overcome female infertility caused by PABPC1L dysfunction, but additional experiments should be pursed for evaluating effectiveness to embryonic development. We have added these sentences to the Discussion section (Line 407-412).

Reference:

1. Guzeloglu-Kayisli O, Lalioti MD, Aydiner F, Sasson I, Ilbay O, Sakkas D, Lowther KM, Mehlmann LM, Seli E (2012) Embryonic poly(A)-binding protein (EPAB) is required for oocyte maturation and female fertility in mice. *Biochem J* 446: 47-58

2. Lowther KM, Mehlmann LM (2015) Embryonic Poly(A)-Binding Protein Is Required During Early Stages of Mouse Oocyte Development for Chromatin Organization, Transcriptional Silencing, and Meiotic Competence. *Biol Reprod* 93: 43

Minor remarks

- Line 28: I would not describe it as a common cause

Response: We have corrected it and described it as “one of the important causes of female infertility” (Line 30).

- Line 115: PB1 oocyte is not a commonly used term, compared to GV and MI/MII oocytes, so please revise this

Response: As suggested, we have changed the all term “PB1 oocyte” in this manuscript to the commonly used term “MII oocyte”.

- Line 145: "The affected individual in family 2 carried the compound heterozygous variants consisting of a missense variant c.410C>T (p.Ser137Phe) and a frameshift insertion variant c.956_957insA (p.Ile320Asnfs*122)." The sentence might need some restructuring. An individual carrying the heterozygous variants consisting of ... feels as incorrect use of terminology.

Response: We have modified the sentence to “The affected individual in family 2 carried the compound heterozygous variants c.410C>T (p.Ser137Phe) and c.956_957insA (p.Ile320Asnfs*122)” (Line 139-141).

- Line 164: "We measured the expression of PABPC1L mRNA and found that PABPC1L was highly expressed in human immature GV and MI oocytes as well as in the lung, but was poorly expressed in mature MII oocyte, early embryos, and other somatic tissues (Fig 1D), suggesting an important role for PABPC1L in human oocyte maturation." Clarify in the manuscript that this data was obtained in control samples.

Response: We have clarified this data was obtained in control samples both in Results (Line 156-157) and Materials and Methods (Line 467) sections.

- Line 170: "To evaluate the functional effects of the identified pathogenic variants in vitro, we first performed immunoblot analysis in HeLa cells transfected with WT or mutant PABPC1L constructs." Clarify in the manuscript what type of constructs: plasmid delivery

Response: We have clarified all the type of constructs in the manuscript.

- Line 248: Here, I would first describe the data more generalized and only towards the end of the paragraph start focussing on the Mos-MAPK, to show that you work from generalized interpretation to targeted interpretation. Clarify a bit more why you focus on the Mos-MAPK in particular, and not the other highly differentially expressed genes.

Response: According to your suggestion, we first described the RNA-seq data more generalized as following "Gene expression levels were assessed as FPKM, and all replicates showed high correlations (Fig 5A). Compared to WT zygotes, 1,933 transcripts were downregulated and 1,952 transcripts were upregulated in G97D zygotes, while 872 transcripts were downregulated and 618 transcripts were upregulated in S137F zygotes (Fig 5B). Furthermore, Venn diagrams showed that there were 344 co-upregulated transcripts and 457 co-downregulated transcripts in the two groups (G97D vs. WT and S137F vs. WT) (Fig 5C). GO analysis revealed that the co-upregulated genes were mainly involved in positive regulation of mitogen-activated protein kinase (MAPK) cascade pathways, phosphorylation, embryonic development, etc, while the co-downregulated genes were involved in translation, cell cycle, meiotic spindle organization, etc. (Fig 5D)".

Next, we started to focus on Mos-MAPK pathway and added more descriptions to clarify why we focus on this pathway as "we first analyzed the differentially expressed genes in G97D zygotes and mainly focused on oocyte-specific genes. We found that *Mos*, an upstream activator of MAPK cascade (Dupre *et al*, 2011), was the only oocyte-specific gene among the top 30 differentially expressed genes (Dataset EV1, Fig 5B). Then we checked the expression of *Mos* in S137F zygotes and observed that it was also up-regulated (Dataset EV1, Fig 5B). The up-regulation of *Mos* in KI zygotes is consistent with the activation of MAPK cascade revealed in GO analysis. The above RNA-seq analysis results strongly suggest that Mos-MAPK pathway is abnormally activated in KI zygote. In addition, studies have indicated that abnormal activation of Mos-MAPK pathway in embryo led to cleavage arrest (Haccard *et al*, 1993; Sagata *et al*, 1989; Verlhac *et al*, 2000), which is similar to the phenotype of early embryonic arrest in KI mice". We have added all the relevant description to the Result section (Line 242-264).

- Line 297: "The infertile phenotype can be explained by abnormal activation of the Mos-MAPK pathway in zygotes." I believe this statement is too strong. For sure, Mos-MAPK is dysregulated in this model, but this is only one step in the molecular cascade leading to the observe phenotype. Please reconsider this statement. In the discussion, you approach this more carefully, which is better.

Response: We have changed this sentence to "The Mos-MAPK pathway was abnormally activated in the zygotes of KI mice, which may provide an explanation the infertile phenotype"(Line 302-303).

- Line 337: "Furthermore, all embryos obtained from the affected individuals in families 2 and 3 were arrested at an early stage, suggesting that PABPC1L also play an important role in early embryonic development prior to ZGA." I would rather state that zygote cleavage is compromised, given the drop in developmental rate between zygote and 2-cell stage shown in fig 4B.

Response: We are apologized for inadequate description of the results in Fig 4B. At 24 h after IVF, compared with WT group, the percentage of *Pabpc1l* KI zygotes dividing into 2-cell stage was indeed significantly reduced. However, as shown in Fig 4C, the cleavage of KI zygotes was delayed, and most KI zygotes could cleavage in the subsequent *in vitro* culture, but all embryos eventually arrested at an early stage and failed to develop into blastocysts. We have added a detailed description of KI zygote cleavage in the Results section (Line 233-236).

- Line 368: "In addition, Pabpc1l KI mice harbouring homozygous missense variants had a milder phenotype than affected individuals in families 1 and 2 harboring compound heterozygous variants consisting of a missense variant and a truncating variant." I would remove this sentence and keep comparisons between different genotypes within one species. So I would only compare mouse with mouse (so KO mouse with KI mouse) and human with human (missense with nonsense).

Response: Thank you for the suggestion, we have removed this sentence and kept comparisons between different genotypes within one species.

- Discussion: You identified novel pathogenic variants in PABPC1L leading to the observed phenotype, but as mentioned in the introduction, the genetic basis and the mechanisms involved in the majority of affected individuals remain unknown. To the discussion, you could add the frequency/pick-up rate of variants in the PABPC1L gene and add some speculation on the missing genetic basis in the majority of patients. In what type of pathways could we still find novel pathogenic variants?

Response: In this study, we identified five individuals with bi-allelic variants in *PABPC1L*, which accounts for 0.26% of our cohort of 1898 infertile woman (1394 with oocyte maturation arrest and 504 with abnormalities in fertilization and early embryonic development). In addition, we also found other candidate pathogenic genes through bioinformatics analysis, but the pathogenicity of these mutant genes needs to be further verified by *in vitro* experiments and the generation of knockout or patient-derived mutated animal models. Nevertheless, the genetic basis involved in the majority of affected individuals remain unknown, which requires extensive exploration using different research strategies. Previous studies have shown that gene-based burden test is an effective strategy for finding novel pathogenic genes in many other disorders (Cirulli *et al*, 2015; Guo *et al*, 2018; Malik *et al*, 2021). By using the strategy, *LHX8* and *KPNA7* variants were identified to cause female infertility (Wang *et al*, *J Clin Invest*, 2023; Zhao *et al*, *Genet Med*, 2022). We believe that applying gene-based burden test will reveal more genetic basis of female infertility. In addition, there is a good understanding of the functional impact of protein-coding variants in female infertility (Jiao *et al*, 2021), but less understanding of variants in non-coding regions, which accounts for 98% of the human genome. In the last decade, many studies have identified that variants in non-coding regions cause different diseases (Turro *et al*, 2020; Wakeling *et al*, 2022; Wright *et al*, 2021), which suggests that the analysis of non-coding regions in female infertility patients with unknown causes is also a powerful

strategy to find novel pathogenic variants. We have added the relevant description to the Discussion section (Line 413-433).

- Line 541: "Clean reads were aligned to the human genome hg38 using STAR and StringTie." wasn't RNAseq performed on mouse?
Response: We have modified “human genome hg38” to “mouse genome mm10” in Materials and Methods sections (Line 593).

- Line 558: The journal doesn't require you to deposit the raw sequencing data in a database?
Response: We have deposited the raw sequencing data in the Genome Sequence Archive in National Genomics Data Center, China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA009502) that are publicly accessible at https://bigd.big.ac.cn/gsa/browse/CRA009502.

Referee #3 (Remarks for Author):

This interesting and well-written manuscript by Wang et al identifies several infertile woman who exhibit defective oocyte development, and find that they bear biallelic mutations in the very interesting gene PABPC1L. In most cases, pedigree genotyping was possible, enabling the authors to reveal phasing and a recessive mode of inheritance. This paper is distinguished by the validation experiments performed by the authors. They performed various assays tailored to the allele type (e.g., mismatches vs premature stops), including transfection of mutant constructs into HeLa cells to confirm predicted protein truncations and functional defects in reporter gene translation or RNA binding. Critically, the authors generate mouse models for the nonsynonymouse alleles, and find that they cause infertility phenotypes that essentially confirm the pathogenicity of the human alleles (and reveal subtle differences in mouse vs human phenotypes that are probably reflective of species differences).
Response: Thank you for your positive evaluation of this study.

Overall, this is an elegant paper with no significant weaknesses. The data support the conclusions. The experimental confirmations of variant effects set this paper aside from many other reports of infertility-causing alleles with far less evidence. I make only the following 3 minor suggestions.
Response: Thank you for your favorable view and suggestions.

- last sentence of abstract. It isn't clear if the "new marker for genetic diagnosis..." is PABPC1L or MOS mRNA levels. PABPC1L isn't really a "marker" but potential candidate to be screened for causes of infertility.
Response: We have changed this sentence into “add a potential genetic candidate gene to be screened for causes of infertility” (Line 43-44).

- Results section starts abruptly on line 104. Maybe an introductory sentence about the patient pool.

Response: We have added an introductory sentence "To investigate the genetic factors responsible for oocyte maturation arrest, we recruited 1394 infertile female individuals diagnosed with oocyte maturation arrest" at the beginning of the Results section (Line 110-111).

- sequence traces probably unnecessary in Fig. 1 (could go to Supplemental).

Response: Thank you for this important and helpful idea. We have put the sequence traces in Fig EV1.

20th Mar 2023

Dear Dr. Wang,

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

1) We note that you currently have together with you, a total of 3 co-corresponding authors. Is that correct? Do you confirm equal contribution of these 3 people, able to take full responsibility for the paper and its content? While there is no limit per se to the number of co-corresponding authors, 3 is rare, and may not reflect as intended to the community.
2) In the main manuscript file, please do the following:
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- In M&M, please include statement that the experiments with human samples conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.
3) Synopsis:
- Synopsis image: Please resize the image to 550 px-wide x (250-400)-px high and submit a high-resolution jpeg file.
- Please check your synopsis text and image before submission with your revised manuscript. Please be aware that in the proof stage minor corrections only are allowed (e.g., typos).
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5) Please provide a point-by-point letter INCLUDING my comments as well as the reviewer's reports and your detailed responses (as Word file).

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Zeljko Durdevic

Zeljko Durdevic
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Referee #1 (Remarks for Author):

The authors added more experiments to their work allowing to answer the Reviewer's comments.

Referee #2 (Comments on Novelty/Model System for Author):

high quality study

Referee #2 (Remarks for Author):

The authors addressed my concerns adequately, and performed additional experiments like ICSI.

The authors addressed the remaining editorial issues.

29th Mar 2023

Dear Dr. Wang,

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Please read below for additional IMPORTANT information regarding your article, its publication and the production process.

Congratulations on your interesting work,

Zeljko Durdevic

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