THE EMBO JOURNAL

Ex vivo reconstitution of fetal oocyte development in humans and cynomolgus monkeys

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DOI: 10.15252/embj.2022110815

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Review Timeline:

Submission Date:	31st Jan 22
Editorial Decision:	25th Mar 22
Revision Received:	28th May 22
Editorial Decision:	24th Jun 22
Revision Received:	28th Jun 22
Accepted:	29th Jun 22

Editor: Daniel Klimmeck

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 25th Mar 2022

Dear Mitinori,

Thank you for submitting your manuscript for consideration by the EMBO Journal and your patience with our response. Your manuscript has now been seen by three referees with expertise in developmental biology and single-cell analysis, whose comments are shown below.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. Please carefully consider their input regarding complementary data analyses required and revised presentation of the results.

As you know, it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

We have recently established Structured Methods as a new format for the Materials and Methods of articles published at EMBO Press. Adhering to this format is optional for research articles. However, considering the strong methodological aspect of your study, we would strongly encourage you to use it. Specifically, the Material and Methods section should include a Reagents and Tools Table (listing key reagents, experimental models, software and relevant equipment and including their sources and relevant identifiers) followed by a Methods and Protocols section in which we encourage the authors to describe their methods using a step-by-step protocol format with bullet points. More information on how to adhere to this format as well as downloadable templates (.doc or .xls) for the Reagents and Tools Table can be found in the author guidelines of our sister journal Molecular Systems Biology
https://www.embopress.org/page/journal/17444292/authorguide#methodguide

An example of a paper with Structured Methods can be found here:
https://www.embopress.org/doi/full/10.15252/embj.2018100300

We encourage you to be even more explicit in adding details on the experimental procedures, as this should be valuable in ensuring reproducible application if the approach.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Kind regards,

Daniel

Daniel Klimmeck, PhD
Senior Editor
The EMBO Journal

Instruction for the preparation of your revised manuscript:

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.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point response to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/Author Checklist%20-%20EMBO%20J-1561436015657.xlsx). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/14602075/authorguide#datadeposition).
In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.
*** Note - All links should resolve to a page where the data can be accessed. ***

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data can be provided as individual .xls or .csv files (including a tab describing the data). For 'blots' or microscopy, uncropped images should be submitted (using a zip archive or a single pdf per main figure if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at .

9) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online (see examples in https://www.embopress.org/doi/10.15252/embj.201695874). 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.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: .

- Additional Tables/Datasets should be labelled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

10) When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

11) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

Further information is available in our Guide to Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (23rd Jun 2022). Please discuss the revision progress ahead of this time with

the editor if you require more time to complete the revisions. Use the link below to submit your revision:

Link Not Available

Referee #1:

This a very through and comprehensive dynamic characterisation of foetal oocyte development. It will serve as a molecular and cellular atlas of oocyte maturation in vivo and in vitro of broad interest for the developmental biology community. The authors have done an excellent job both at the stage of setting up the experimental conditions and at the level of analysis. I support the publication of this manuscript, after the following minor comments have been addressed (no experiments are required):

1. Reconstitution of foetal oocyte development in vitro: in their experimental protocol the authors disaggregate the ovary into single cells, which are then reaggregated to form spheres. Is the disaggregation step really necessary? I imagine this step would be really useful when trying to co-culture somatic ovarian cells with human oogonia-like cells. But, is it really necessary if no exogenous cells are added? It is also not clear whether upon reaggregation the characteristic anatomical structure of the ovary (described in figure 1) is reformed. The authors just mention that the core is not very healthy. Do they see an outer cortex and an inner cortex? If not, this would mean oocyte maturation can happen in the absence of an appropriate ovarian anatomy.

2. Comparisons between in vivo and in vitro: the authors conclude that the transcriptome of the in vitro cells was indistinguishable from the transcriptome of the in vivo cells. This is not clearly shown in the manuscript. Has a differential gene expression analysis been done? Are there no genes differentially expressed between the in vitro cells and their in vivo counterparts? Figure 4J shows a cluster of unclassified cells, and some of the in vitro cells are assigned an unclassified identity. What is this cluster exactly? It is of course important to emphasise the similarities between in vivo and in vitro but the authors should also acknowledge potential differences.

3. Maturation dynamics in xenotransplantation and in vitro culture: based on the data shown in figures 4L and 4K it seems that the xenotransplantation samples do not really progress with time. While the progression is clear in the in vitro cultured samples, in the case of xenotransplantation there is a complete mix of identities in the different time points analysed. The authors note that in vitro the time required for differentiation is longer, but this does not seem to be the case based on these two panels.

4. Panel 4K: what was the criteria to select those specific genes? Could the authors plot the genes that characterise the clusters identified in panel 4H?

5. Discussion: the authors have previously shown that human primordial germ cell like cells (hPGCLCs) can be differentiated in vitro into oogonia. I think the next natural step would be to apply the protocol describe in this manuscript to attempt further maturation of the hPGCLC-derived oogonia. This is something that perhaps the authors could mention in the discussion.

6. In certain parts the paper looks like a catalog of genes. I think the text could be simplified by providing some of the information on markers genes as a supplementary table instead.

Referee #2:

Summary: In this manuscript, the authors optimized an in vitro culture condition for fetal oocyte development both in humans and monkeys, showing that mitotic oogonia enter into and complete the first meiotic prophase to form primordial follicles. They demonstrated that fetal oocyte development in both species can be faithfully reconstituted in vitro based on cytological and single-cell transcriptomic analysis. Furthermore, by comparing single-cell transcriptomes among humans, monkeys and mice, they identified evolutionary conserved and primate-specific transcriptional and cellular programs during fetal oocyte development.

The present manuscript is potentially important because it provides new insights into primate-specific transcriptional and cellular programs during fetal oocyte development. However, their main claims based on transcriptomic analysis are not well supported by data presented.

Major points:
1. Fig. EV3E: The authors should provide the proportion of the predicted cell cycle phase (G1, S, and G2/M) of cells across cell types, and check whether the cell cycle assignments are consistent with their conclusions based on marker expression and GO enrichment analysis.
2. Fig. 4G: The trajectory graph should be provided. The heatmap showing the pseudo-temporal expression patterns of HVGs should be also provided, and this should be consistent with Fig. 4H.

3. Fig. 4J-L: The main claim that cy fetal germ cells undergo appropriate transcriptomic maturation in vitro" was not well supported. The newly generated scRNA-seq data have too small number of cells to be properly analyzed. To address this issue, the in vivo scRNA-seq data in Fig. 4D should be served as a reference map to understand all the scRNA-seq data. For this, each data should be projected into the UMAP in Fig. 4D based on the transcriptomic similarity. The pseudo-temporal expression patterns of genes among data should be compared in a more systematic way.
4. Fig. EV4F: A substantial number of cells at 12/15 w-ivc were merged with cluster 4. What is the characteristics of these cells?
5. Fig. 5E-G: This human in vitro scRNA-seq data should be projected into the reference map in Fig. 4D and the pseudo-temporal expression patterns should be carefully compared.
6. Fig. 6D: This difference between humans and mice might be driven by the sampling issue. It seems that human cells were enriched in the mitotic compartment, but mouse cells were evenly distributed across cell types. The authors should carefully address this issue.
7. Fig. 6E: The authors should evaluate the robustness of their claim by using all expressed orthologous genes common to the three species.
8. Fig. 6F: The expression patterns of 83 genes showing specific expression changes in humans and monkeys should be also examined in the other mouse data to confirm the robustness of this finding (Zhao et al. and Ge et al.)
9. Fig. 7D: This analysis might be also affected by the sampling issue because each cell type has the different number of cells. I'm wondering whether the main conclusion is not affected if we fix the number of cells across cell types.

Minor points:
1. Fig. 3E: The time information was not well distinguished. It would be better to present this like Fig. 3B.
2. Fig. EV4F: The clusters should be renamed to avoid the confusion arisen from clusters in Fig. EV4A.

Referee #3:

In the manuscript entitled "Ex vivo reconstitution of fetal oocyte development in humans and monkeys", Ken Mizuta and colleagues deeply studied the female germ line development in primates using Cynomolgus monkey and human models. The study provides an extremely well detailed characterization of oogenesis in Cy monkeys in vivo and ex vivo by both IF and transcriptomics. With an extensive analysis of different culture medium components, cell culture coating and set-up, the authors have successfully recapitulated in vitro maturation of Cy fetal ovaries. They then adapted their protocol to human fetal ovaries with success. Transcriptomic analysis of human, monkey and mouse datasets (either produced by the authors or from public datasets) showed important evolutionary-conserved and primate-specific as well as species-specific genes involved in oogonia development.
One of the specificities of female germline development is X-chromosome reactivation (XCR) and question remains open if this is linked to excess of X-linked gene expression, with contradictory studies in humans (Sangrithi et al., 2017 and Chitiashvili et al., 2020). Based on their datasets and public ones, the authors analysed X:autosome ratio and concluded little to no X-linked gene excess despite XCR, due to erasure of X-chromosome upregulation. This is significant results for the X-chromosome regulation field. However, before formally supporting this conclusion, the authors should reanalyse the datasets from Sangrithi study with their bioinformatics pipeline. Furthermore, how to measure X:A ratios has been debated here (Kharchenko et al., 2011). It would then be important to support the manuscript conclusions with X:A ratios measurement based on these recommendations (Kharchenko et al, 2011).
Minor concerns:
- Number of analysed cells and/or number of biological replicates are often missing and should be given in figure legends (i.e. Fig2D, 3F,EV1, EV2). Number of performed xenotransplantation experiments should be stated.
- Introduction of X-chromosome regulation (and then Figure 7) should be included in the introduction section.
- Colour choices of Late pachytene and diplotene make it difficult to read, especially on printed version.

<u>Rebuttal: EMBOJ-2022-110815</u>
We would like to sincerely thank the Reviewers for their constructive comments, which we have used as the basis for revising our manuscript.

Reviewers' Comments:
<u>Reviewer: 1</u>
This a very through and comprehensive dynamic characterisation of foetal oocyte development. It will serve as a molecular and cellular atlas of oocyte maturation in vivo and in vitro of broad interest for the developmental biology community. The authors have done an excellent job both at the stage of setting up the experimental conditions and at the level of analysis. I support the publication of this manuscript, after the following minor comments have been addressed (no experiments are required):

Response 1. We sincerely thank the Reviewer for the encouraging comments.

1. Reconstitution of foetal oocyte development in vitro: in their experimental protocol the authors disaggregate the ovary into single cells, which are then reaggregated to form spheres. Is the disaggregation step really necessary? I imagine this step would be really useful when trying to co-culture somatic ovarian cells with human oogonia-like cells. But, is it really necessary if no exogenous cells are added?

Response 2. We agree with the Reviewer that an *ex vivo* reconstitution of cynomolgus monkey (cy) or human (h) fetal oocyte development itself does not require the dissociation/re-aggregation steps of fetal ovaries. As pointed out by the Reviewer, a key reason why we adopted these steps was to apply the protocol to the re-aggregation with cy/h oogonia-like cells induced from cy/h pluripotent stem cells (PSCs) in future experiments. In addition, the adoption of dissociation/re-aggregation steps has the following advantages: 1) the dissociation of fetal ovaries into single cells allows us to prepare frozen stocks with an appropriate cell number, which in turn makes systematic exploration of the culture conditions feasible; 2) the dissociation of fetal ovaries into single cells allows us to selectively isolate the required cell populations, e.g., granulosa cells, for subsequent analysis. We provided an explanation of these points in the revised manuscript (**INTRODUCTION** section, 4th paragraph).

It is also not clear whether upon reaggregation the characteristic anatomical structure of the ovary (described in figure 1) is reformed. The authors just mention that the core is not very healthy. Do they see an outer cortex and an inner cortex? If not, this would mean oocyte maturation can happen in the absence of an appropriate ovarian anatomy.

Response 3. The typical width of the cy fetal ovarian cortex *in vivo* is ~450 µm, while the cy/h reconstituted ovaries (rOvaries) we generated are ~250 µm in radius, and we did not recognize an outer/inner cortex-like structural stratification in cy/h rOvaries. On the other hand, we did observe ovarian cord-like structures in cy/h rOvaries. We provided additional histological/immunofluorescence (IF) images of cy rOvaries to

clarify this point and relevant discussion in the revised manuscript (Appendix Fig S1, **"Cy fetal oocyte development"** section, 2nd paragraph; **"Cy fetal oocyte development in xenotransplanted cy rOvaries"** section, 2nd paragraph; **"Reconstitution of cy fetal oocyte development *in vitro*"** section, 2nd paragraph).

2. Comparisons between in vivo and in vitro: the authors conclude that the transcriptome of the in vitro cells was indistinguishable from the transcriptome of the in vivo cells. This is not clearly shown in the manuscript. Has a differential gene expression analysis been done? Are there no genes differentially expressed between the in vitro cells and their in vivo counterparts?

Response 4. We would like to sincerely thank the Reviewer for this comment. In response to this point, we performed a more careful comparison of the gene expression between cy fetal oocytes *in vivo* and *in vitro* using the SC3-seq data, because the SC3-seq detects larger numbers of genes and shows better quantitative performance than the 10X scRNA-seq (Nakamura, Yabuta et al., 2015) (Appendix Fig S5A and Fig EV3C in the revised manuscript). We found that the correlation coefficients between *in vivo* and *in vitro* RA-responsive/meiotic/oogenic cells in all the expressed genes were 0.968/0.968/0.982, respectively (Appendix Fig S5B in the revised manuscript). The differentially expressed genes (DEGs) (those with more than 4-fold expression differences (Nakamura et al., 2015)) between *in vivo* and *in vitro* RA-responsive/meiotic/oogenic cells were few in number, but included some key genes for oocyte development, including *ZGLP1*, *REC8* (RA-responsive), *SOHLH1*, *NOBOX* (meiotic), *NLRP4*, and *ZP4* (oogenic) (Table EV4). Examination of their expression along the pseudo-time developmental trajectory suggested that while the differential expression of *SOHLH1*, *NOBOX* (meiotic), *NLRP4*, and *ZP4* (oogenic) may result from a difference in meiotic/oogenic stages in the cell populations used for the comparison, the differential expression of *ZGLP1*and *REC8* (RA-responsive) may reflect a significant difference (Appendix Fig S5C), which could explain why the pre-leptotene to leptotene transition may be a rate-limiting step under the current culture condition (Fig 3G). Collectively, these facts lead us to conclude that *in vivo* and *in vitro* fetal oocytes are highly similar in gene expression, but the up-regulation of key genes for the pre-leptotene to leptotene transition may not be optimal under the current *in vitro* culture condition. We provided relevant data and discussion in the revised manuscript (Appendix Fig S5, Table EV4, **"Transcriptome dynamics for cy fetal ovary development *in vivo*, in xenotransplantation, and *in vitro*"** section, 4th paragraph; **DISCUSSION** section, 3rd paragraph).

Figure 4J shows a cluster of unclassified cells, and some of the in vitro cells are assigned an unclassified identity. What is this cluster exactly? It is of course important to emphasise the similarities between in vivo and in vitro but the authors should also acknowledge potential differences.

Response 5. The unclassified *in vivo* cells (Fig 4D: labelled as U) showed significantly

lower levels of nUMI and nGenes (Fig EV3C), and exhibited gene-expression profiles highly correlated with those of pre-leptotene/leptotene cells (Fig 4I), and we assume that these cells were apoptotic cells at the pre-leptotene/leptotene stage, which is briefly described in the original manuscript. In good agreement with this idea, we detected germ cells with apoptotic markers (cCAS3 and cPARP) at a relatively high ratio at 10 and 12 wpf (Fig EV1F), which is the time range when mitotic germ cells initiate differentiation into pre-leptotene/leptotene cells (Fig 1G), and the dynamics of the emergence of apoptotic cells was somewhat similar to that of RA-responsive cells (pre-leptotene/leptotene cells) during fetal oocyte development (Fig 1G). We also detected germ cells with apoptotic markers in cy rOvaries *in vitro* (Fig EV2C), indicating that a fraction of germ cells also undergo apoptosis upon the progression of meiotic prophase *in vitro*. We provided these data and relevant discussion in the revised manuscript (Fig 1G, 4D and I, Fig EV1F, 2C, and 3C, **"Transcriptome dynamics for cy fetal ovary development *in vivo*, in xenotransplantation, and *in vitro*"** section, 1st paragraph).

3. Maturation dynamics in xenotransplantation and in vitro culture: based on the data shown in figures 4L and 4K it seems that the xenotransplantation samples do not really progress with time. While the progression is clear in the in vitro cultured samples, in the case of xenotransplantation there is a complete mix of identities in the different time points analysed. The authors note that in vitro the time required for differentiation is longer, but this does not seem to be the case based on these two panels.

Response 6. The reason why the maturation dynamics may not be properly represented in the SC3-seq data for the xenotransplantation experiments (Fig 4K and L) was that the number of cells analyzed in this experiment was small, in part because cy fetal ovaries are a limited material, and it was somewhat technically difficult to dissociate transplanted cy rOvaries into single cells. On the other hand, we analyzed the maturation dynamics of cy fetal oocytes under the xenotransplantation condition primarily through histological analyses, which allowed us to analyze the entire cy rOvary structures and revealed that, upon xenotransplantation, cy fetal oocyte development and the meiotic prophase I proceed in an apparently normal fashion, although the developmental progression seemed somewhat slower compared to that *in vivo* (Fig 2B−D). We provided an appropriate explanation in the revised manuscript (**"Transcriptome dynamics for cy fetal ovary development *in vivo*, in xenotransplantation, and *in vitro*"** section, 4th paragraph).

4. Panel 4K: what was the criteria to select those specific genes? Could the authors plot the genes that characterise the clusters identified in panel 4H?

Response 7. For Fig 4K, we selected a minimum set of genes that differentially label mitotic, RA-responsive, meiotic, and oogenic cells.

In response to the Reviewer's comment, we analyzed the expression of highly variable

genes (HVGs) identified by the scRNA-seq analysis with the 10X Chromium platform (Fig 4H) in the scRNA-seq data generated with the SC3-seq method along the pseudo-time trajectory, as shown in Fig 4G. We also performed the same analysis for the human SC3-seq data using the human orthologues of the HVGs. These analyses revealed similar gene-expression dynamics of the HVGs and their orthologues in the SC3-seq data for cy/h fetal oocyte development *in vivo* as well as *in vitro* (Appendix Fig S4A and B), underscoring the consistency between the scRNA-seq analyses with the 10X Chromium platform and the SC3-seq as well as the similarity of cy/h fetal oocyte development *in vivo* and *in vitro*. We provided these data and relevant discussion in the revised manuscript (Appendix Fig S4A and B, **"Transcriptome dynamics for cy fetal ovary development *in vivo*, in xenotransplantation, and *in vitro*"** section, 4th paragraph; **"Reconstitution of human fetal oocyte development *in vitro*"** section, 3rd paragraph).

5. Discussion: the authors have previously shown that human primordial germ cell like cells (hPGCLCs) can be differentiated in vitro into oogonia. I think the next natural step would be to apply the protocol describe in this manuscript to attempt further maturation of the hPGCLC-derived oogonia. This is something that perhaps the authors could mention in the discussion.

Response 8. We would like to thank the Reviewer for this comment. We provided relevant statements in the revised manuscript (**INTRODUCTION** section, 4th paragraph; **DISCUSSION** section, 5th paragraph).

6. In certain parts the paper looks like a catalog of genes. I think the text could be simplified by providing some of the information on markers genes as a supplementary table instead.

Response 9. In response to the Reviewer's suggestion, we have minimized or entirely removed multiple gene lists in the relevant parts of the main text. The full gene lists are still available in Table EV3 and EV5.

<u>Reviewer: 2</u>

Summary: In this manuscript, the authors optimized an in vitro culture condition for fetal oocyte development both in humans and monkeys, showing that mitotic oogonia enter into and complete the first meiotic prophase to form primordial follicles. They demonstrated that fetal oocyte development in both species can be faithfully reconstituted in vitro based on cytological and single-cell transcriptomic analysis. Furthermore, by comparing single-cell transcriptomes among humans, monkeys and mice, they identified evolutionary conserved and primate-specific transcriptional and cellular programs during fetal oocyte development.

The present manuscript is potentially important because it provides new insights into primate-specific transcriptional and cellular programs during fetal oocyte development.

However, their main claims based on transcriptomic analysis are not well supported by data presented.

Major points:
1. Fig EV3E: The authors should provide the proportion of the predicted cell cycle phase (G1, S, and G2/M) of cells across cell types, and check whether the cell cycle assignments are consistent with their conclusions based on marker expression and GO enrichment analysis.

Response 1. To address the Reviewer's request, we assigned the cell-cycle phase of cy germ cells using the "Cell-Cycle Scoring" function of the Seurat package based on the strategy described in Tirosh *et al*. (Tirosh, Izar et al., 2016). Since our datasets include oocytes in meiotic prophase I, we used the orthologous genes from the annotation list of the meiotic cells in Shimada *et al*. (Shimada, Koike et al., 2021) to assign the meiotic prophase I. Because this widely used scoring method does not distinguish meiotic from mitotic G1/S/G2 phases, the annotated cell-cycle phases correspond to the sum of the mitotic and meiotic phases (Appendix Fig S2B), which is highly consistent with our conclusion shown in Fig EV3B. Additionally, in accord with our annotation, this result assigned both mitotic and pre-leptotene 1/2/3 to G1, S, and G2/M, despite relatively minor annotation differences, which would presumably be because the Seurat method uses markers defined in human and mouse somatic cells (Tirosh et al., 2016) (Appendix Fig S2A−C). We provided these data and relevant discussion in the revised manuscript (Appendix Fig S2A−C, **"Transcriptome dynamics for cy fetal ovary development *in vivo*, in xenotransplantation, and *in vitro*"** section, 2nd paragraph).

2. Fig 4G: The trajectory graph should be provided. The heatmap showing the pseudo-temporal expression patterns of HVGs should be also provided, and this should be consistent with Fig 4H.

Response 2. In response to the Reviewer's request, we drew a cell-fate trajectory line using the Monocle 3 package and displayed it on the UMAP in Fig 4G. We then defined the variable genes based on the Moran's ***I*** statistic using the Monocle 3 package and displayed the expression patterns of these genes as well as the HVGs defined in Fig 4H along the pseudo-time developmental trajectory, both of which provided an outcome consistent with the data in Fig 4H (Appendix Fig S3A and B). We added these data and relevant discussion to the revised manuscript (Fig 4G, Appendix Fig S3A and B, **"Transcriptome dynamics for cy fetal ovary development *in vivo*, in xenotransplantation, and *in vitro*"** section, 3rd paragraph).

3. Fig 4J-L: The main claim that cy fetal germ cells undergo appropriate transcriptomic maturation in vitro" was not well supported. The newly generated scRNA-seq data have too small number of cells to be properly analyzed. To address this issue, the in vivo scRNA-seq data in Fig 4D should be served as a reference map to understand all the scRNA-seq data. For this, each data should be projected into the UMAP in Fig 4D

based on the transcriptomic similarity. The pseudo-temporal expression patterns of genes among data should be compared in a more systematic way.

Response 3. Please note that SC3-seq is a highly sensitive scRNA-seq method that allows a quantitative comparison of single-cell gene expression profiles with a minimum of ~four−eight single cells (Nakamura et al., 2015). Accordingly, we performed a more careful comparison of the gene expression between cy fetal oocytes *in vivo* and *in vitro* using the SC3-seq data. We found that the correlation coefficients between *in vivo* and *in vitro* RA-responsive/meiotic/oogenic cells in all the expressed genes were 0.968/0.968/0.982, respectively (Appendix Fig S5B in the revised manuscript). The differentially expressed genes (DEGs) (those exhibiting more than 4-fold expression differences (Nakamura et al., 2015)) between *in vivo* and *in vitro* RA-responsive/meiotic/oogenic cells were few in number, but included some key genes for oocyte development, including *ZGLP1*, *REC8* (RA-responsive), *SOHLH1*, *NOBOX* (meiotic), *NLRP4*, and *ZP4* (oogenic) (Table EV4). Examination of their expression along the pseudo-time developmental trajectory suggested that while the differential expression of *SOHLH1*, *NOBOX* (meiotic), *NLRP4*, and *ZP4* (oogenic) may result from a difference in meiotic/oogenic stages in the cell populations used for the comparison, the differential expression of *ZGLP1*and *REC8* (RA-responsive) may reflect a significant difference (Appendix Fig S5C), which could explain why the pre-leptotene to leptotene transition may be a rate-limiting step under the current culture condition (Fig 3G). Collectively, these facts lead us to conclude that *in vivo* and *in vitro* fetal oocytes are highly similar in gene expression, but the up-regulation of some genes for the pre-leptotene to leptotene transition may not be optimal under the current *in vitro* culture condition. We provided relevant data and discussion in the revised manuscript (Appendix Fig S5, Table EV4, **"Transcriptome dynamics for cy fetal ovary development *in vivo*, in xenotransplantation, and *in vitro*"** section, 4th paragraph) (please also see **Response 4** to the **Reviewer 1**).

Additionally, according to the Reviewer's comment, we projected the query data, i.e., 10X scRNA-seq data for *in vitro* cy germ cells at 12/15 w-ivc, onto the mutual nearest neighbor (MNN)-corrected PCA space constructed from the reference 10X scRNA-seq data for *in vivo* cy germ cells, which demonstrated a clear overlap of the *in vitro* cells with the *in vivo* cells (Fig R1A). On the other hand, by performing the same analysis for *WT1*$^{+}$ cy pre-granulosa cells, we noted that this suggested method, i.e., the projection of the *in vitro* query data onto the *in vivo* reference data, results in masking the differences between the query and reference data on the UMAP space (Fig R1B). We therefore decided to use the original method and present the original data in the revised manuscript. We hope that our decision meets the approval of the Reviewer.

Furthermore, as described above in **Response 2**, we examined the pseudo-temporal expression patterns of the HVGs shown in Fig 4H and their human orthologues for both 10X scRNA-seq and SC3-seq data for cy/h fetal oocyte development *in vivo* and *in vitro*,

which demonstrated highly similar gene-expression changes along the pseudo-time developmental trajectory during cy/h fetal oocyte development *in vivo* and *in vitro* (Appendix Fig S3 and S4) (please also see **Response 7** to **Reviewer 1**). Taken together, as stated in the original manuscript, these findings led us to conclude that cy fetal germ cells undergo appropriate transcriptome maturation in cy rOvaries *in vitro*. We provided relevant data and discussion in the revised manuscript (Fig 4D and 4J, Appendix Fig S3−5, Table EV4, **"Transcriptome dynamics for cy fetal ovary development *in vivo*, in xenotransplantation, and *in vitro*"** section, 3rd/4th paragraph; **"Reconstitution of human fetal oocyte development *in vitro*"** section, 3rd paragraph).

4. Fig EV4F: A substantial number of cells at 12/15 w-ivc were merged with cluster 4. What is the characteristics of these cells?

Response 4. We would like to thank the Reviewer for this constructive comment. We performed further analysis of cluster d in Appendix Fig S6A (cluster 4 in the original manuscript; please also see **Response 10** below), and found that this cluster consists not only of a subset of granulosa cells in cy rOvaries at 12/15 w-ivc (~50%), but also of similar cells in cy fetal ovaries *in vivo* (~50%), particularly those at 16 wpf (Appendix Fig S6B). The HVGs in this cluster were enriched with genes for "extracellular matrix organization," "angiogenesis," and "cell-cell adhesion" (Appendix Fig S6C). While the identity and the function of this granulosa-cell subset are unclear (and we consider their clarification beyond the scope of this manuscript), they are present in both cy rOvaries *in vitro* and fetal ovaries *in vivo*, providing further evidence that the *in vitro* culture of cy rOvaries recapitulates the fetal ovary development *in vivo*. We provided relevant data and discussion in the revised manuscript (Appendix Fig S6, **"Transcriptome dynamics for cy fetal ovary development *in vivo*, in xenotransplantation, and *in vitro*"** section, 5th paragraph).

5. Fig 5E-G: This human in vitro scRNA-seq data should be projected into the reference map in Fig 4D and the pseudo-temporal expression patterns should be carefully compared.

Response 5. Please see **Response 3**.

6. Fig 6D: This difference between humans and mice might be driven by the sampling issue. It seems that human cells were enriched in the mitotic compartment, but mouse cells were evenly distributed across cell types. The authors should carefully address this issue.

Response 6. We would like to thank the Reviewer for this thoughtful comment. In accord with the Reviewer's suggestion, we first attempted to create datasets consisting of evenly distributed cell populations by random selection, but due to a low number of human fetal oocytes after the zygotene stage in the human 10X dataset (Chitiashvili, Dror et al., 2020), we found that this strategy was not quite effective We therefore

adopted two methodologies: first, we adjusted the cell-type ratios in all the datasets by random selection (Fig R2A), and performed a pseudo-time analysis for each dataset, which revealed that, consistent with our claim based on the original analysis (Fig 6D), humans and monkeys exhibit larger transcriptomic alterations upon the mitotic to pre-leptotene transition than those observed in mice (Fig R2B). Second, to avoid an unpredictable influence of data merging by CCA, we performed the cell-type annotation and pseudo-time analysis on each dataset separately, and this approach also demonstrated a primate-specific major transcriptomic alteration upon the mitotic to pre-leptotene transition (Fig R3A). Additionally, we ran the original analysis pipeline using two other mouse datasets with very different cell-type proportions (Ge, Wang et al., 2021, Zhao, Ma et al., 2020), which gave essentially the same outcome—namely, an overlapping gene-expression profile between mitotic and pre-leptotene/leptotene cells in mice (Appendix Fig S8A and B). These data strongly support our original claim. Accordingly, we decided to present the original analysis as well as that using the two other mouse datasets and the relevant discussion in the revised manuscript (Fig 6A−D, Appendix Fig S8A and B, **"Primate-specific program for fetal oocyte development"** section, 2nd paragraph). We sincerely hope that the Reviewer approves our decision.

7. Fig 6E: The authors should evaluate the robustness of their claim by using all expressed orthologous genes common to the three species.

Response 7. We calculated the correlation coefficients among fetal oocyte cell types using all expressed orthologous genes (12,390 genes) common to the three species (Fig R4). The results were highly consistent with our original data using the 237 HVGs (Fig 6E), although the correlation coefficients were higher overall, which was most likely due to the inclusion of many constantly expressed genes.

8. Fig 6F: The expression patterns of 83 genes showing specific expression changes in humans and monkeys should be also examined in the other mouse data to confirm the robustness of this finding (Zhao et al. and Ge et al.)

Response 8. We have generated heatmap figures showing the expression of the 83 genes with specific expression changes in humans and monkeys as well as the 137 genes with common expression changes in humans, monkeys, and mice in the other two mouse datasets (Ge et al., 2021, Zhao et al., 2020) (Appendix Fig S8C and D), and these new heatmap figures were consistent with the original data in Fig 6F. We added the data and relevant discussion to the revised manuscript (Appendix Fig S8C and D, **"Primate-specific program for fetal oocyte development"** section, 3rd paragraph).

9. Fig 7D: This analysis might be also affected by the sampling issue because each cell type has the different number of cells. I'm wondering whether the main conclusion is not affected if we fix the number of cells across cell types.

Response 9. In response to the Reviewer's comment, we performed the relevant

analysis using the same approach as described above in **Response 6**. Both analyses, i.e., the analysis with the same cell-type ratio for humans, monkeys, and mice (Fig R2C), and the individual data analysis without CCA (Fig R3B) gave an outcome essentially consistent with our original analysis and conclusion (Fig 7D). Additionally, we confirmed the same X:A ratio dynamics along fetal oocyte development when using the other two mouse datasets (Fig EV5D). We provided the data and relevant discussion in the revised manuscript (Fig 7D, Fig EV5D, **"Conserved activities of X chromosomes"** section, 3rd paragraph).

Minor points:
1. Fig 3E: The time information was not well distinguished. It would be better to present this like Fig 3B.

Response 10. We have modified Fig 3E to present the images along the time course by replacing the IF images for SYCP1 and γH2AX at 6 w-ivc with those at 9 w-ivc.

2. Fig EV4F: The clusters should be renamed to avoid the confusion arisen from clusters in Fig EV4A.

Response 11. We have renamed the clusters "Clusters a–n" (Fig EV4F or Appendix Fig S6A). We also moved Fig EV4F/G in the original manuscript to the appendix as Appendix Fig S6A, B, and D in order to streamline the data presentation.

<u>Reviewer: 3</u>
In the manuscript entitled "Ex vivo reconstitution of fetal oocyte development in humans and monkeys", Ken Mizuta and colleagues deeply studied the female germ line development in primates using Cynomolgus monkey and human models. The study provides an extremely well detailed characterization of oogenesis in Cy monkeys in vivo and ex vivo by both IF and transcriptomics. With an extensive analysis of different culture medium components, cell culture coating and set-up, the authors have successfully recapitulated in vitro maturation of Cy fetal ovaries. They then adapted their protocol to human fetal ovaries with success. Transcriptomic analysis of human, monkey and mouse datasets (either produced by the authors or from public datasets) showed important evolutionary-conserved and primate-specific as well as species-specific genes involved in oogonia development.

One of the specificities of female germline development is X-chromosome reactivation (XCR) and question remains open if this is linked to excess of X-linked gene expression, with contradictory studies in humans (Sangrithi et al., 2017 and Chitiashvili et al., 2020). Based on their datasets and public ones, the authors analysed X:autosome ratio and concluded little to no X-linked gene excess despite XCR, due to erasure of X-chromosome upregulation. This is significant results for the X-chromosome regulation field. However, before formally supporting this conclusion, the authors should reanalyse the datasets from Sangrithi study with their bioinformatics pipeline.

Response 1. In response to the Reviewer's comment, we re-analyzed the mouse bulk RNA-seq datasets by Sangrithi *et al*. using our bioinformatics pipeline. Our results were similar to theirs, but we noted that the quality of their data was somewhat more variable: for example, for gonadal somatic cells, which should serve as a control, the Chr3:A ratio ranged from ~1.0−1.5 (ours: ~1.0−1.1), the X:A ratio ranged from ~0.76−1.6 (ours: ~0.82−0.95), and the *Xist* expression levels [$\log_2$(normalized fragment counts+1) value] ranged from ~1.3−3.8 (ours: ~4.1−5.7) (Appendix Fig S9). However, only 2−5 replicates were sampled for germ cells at each embryonic stage: for example, the X:A ratios of two datasets of germ cells at E12.5 were ~0.9, while those of the other two were ~1.2, making it somewhat difficult to draw a definitive conclusion.

In addition to using the data with better quality, we have drawn our conclusion based on two different experimental/analytical methods (10X and SC3-seq/bulk 3'-seq) to evaluate the X:A ratio dynamics in humans, monkeys, and mice under equivalent conditions. In particular, we considered that the cross-species consistency of the 10X data, which involves a huge number of cells, to be highly supportive of our conclusion. We added the data and relevant discussion to the revised manuscript (Appendix Fig S9, **"Conserved activities of X chromosomes"** section, 2nd paragraph; **DISCUSSION** section, 5th paragraph).

Furthermore, how to measure X:A ratios has been debated here (Kharchenko et al., 2011). It would then be important to support the manuscript conclusions with X:A ratios measurement based on these recommendations (Kharchenko et al, 2011).

Response 2. In response to the Reviewer's suggestion, we re-calculated all the data according to the method suggested by *Kharchenko et al*. (2011) (Kharchenko, Xi et al., 2011). Specifically, we excluded genes in the pseudo-autosomal region (PAR) from the analysis and examined stepwise thresholds for excluding lowly expressed genes. As shown in Fig R5A, as the threshold increased, the number of genes used for the analysis declined, and the analysis results came to represent increasingly sparse information, but, critically, the overall dynamics of the X:A ratio remained essentially the same (Fig R5B).

In our original analysis of the 10X data, we also excluded the lowly expressed genes from the analysis, i.e., only genes with their UMIs detected in more than two cells were used for the analysis. As to the RNA-seq/SC3-seq data, we used the 75th-percentile $\log_2$ (normalized read counts+1) values of the expressed genes in individual samples/cells as representative values in the samples/cells, and accordingly, the lowly expressed genes were not included in the analysis. We therefore performed a further analysis with exclusion of the PAR genes and found that the results were essentially the same (Fig R6A−C). These findings strongly support our original claim, and thus we decided to present the original analysis in the revised manuscript. We sincerely hope that the Reviewer approves our decision.

Minor concerns:

- Number of analysed cells and/or number of biological replicates are often missing and should be given in figure legends (i.e. Fig2D, 3F,EV1, EV2). Number of performed xenotransplantation experiments should be stated.

Response 3. We provided precise numbers of cells analyzed and biological replicates, including those for the xenotransplantation experiments, in the relevant figure legends in the revised manuscript.

- Introduction of X-chromosome regulation (and then Figure 7) should be included in the introduction section.

Response 4. In response to the Reviewer's comment, we provided a brief introduction on the X-chromosome regulation and its relevance to Figure 7 in the 2nd paragraph of the **INTRODUCTION** section in the revised manuscript.

- Colour choices of Late pachytene and diplotene make it difficult to read, especially on printed version.

Response 5. In response to the Reviewer's comment, we have changed the color of the diplotene to black for easier distinction throughout the revised manuscript.

LEGENDS TO FIGURES FOR THE REVIEWERS

Figure R1. Projection of the *in vitro* data onto the *in vivo* reference UMAP.

UMAP plots for germ cells (A) and granulosa cells (B) from *in vivo* cy ovaries and *in vitro* cy rOvaries. Query data, i.e., cy germ cells from cultured rOvaries at 12/15 w-ivc, were projected onto the MNN-corrected PCA space, which was the reference constructed from *in vivo* fetal cy germ cells. The procedure consists of 1) cosine-normalization, 2) subtraction of the reference grand center, and 3) transformation by reference eigenvectors. The batch effect between reference and query PCA was corrected on PC1-30 using the "reducedMNN" function ("batchelor" R package) with the default parameters, followed by projection of the query data onto the reference map with the "RunUMAP" function.

Figure R2. Cross-species comparison of fetal oocyte development with 10X scRNA-seq datasets matched for cellular composition ratios.

(A) The numbers of germ cells at each meiotic sub-stage in the human/cy/mouse matched datasets. Cells were randomly selected to match the germ-cell sub-stage composition in each dataset. (B) The distribution of germ cells along the individually calculated species-specific pseudo-time trajectories. (C) The autosome:A and X:A ratios (top), and *XIST*/*Xist* expression transitions (bottom) during fetal oocyte development in the three species. M, mitotic; PL, pre-leptotene; L, leptotene; Z, zygotene; P, pachytene; D, diplotene.

Figure R3. Cross-species comparison of fetal oocyte development by individual analyses of the 10X scRNA-seq datasets.

(A) The distribution of germ cells along the pseudo-time trajectories. The meiotic sub-stages were individually annotated for each dataset. (B) The autosome:A and X:A ratios (top), and *XIST*/*Xist* expression transitions (bottom) during fetal oocyte development in the three species. The meiotic sub-stages were individually annotated for each dataset. M, mitotic; PL, pre-leptotene; L, leptotene; Z, zygotene; P, pachytene; D, diplotene.

Figure R4. Correlation of gene expression during fetal oocyte development in humans, monkeys, and mice.

Heatmap of the Pearson correlation coefficients of the average expression levels of all expressed genes (1-1-1 orthologous, 12,390 genes) among the meiotic sub-stages in humans, monkeys, and mice. The color-coding for the germ cell stage is as indicated.

Figure R5. X:A ratios during fetal oocyte development analyzed with various gene expression thresholds.

All 10X scRNA-seq datasets were reanalyzed following the method discussed in Kharchenko *et al*. (2011) (Kharchenko et al., 2011). (A) The numbers of the genes on

the chromosome (chr.) X, A, and all autosomes with the indicated thresholds for excluding low expression genes. (B) The autosome:A and X:A ratios during fetal oocyte development *in vivo* in the three species analyzed with four different gene thresholds. Each threshold is defined in Fig R5A.

Figure R6. Re-analyses of the X:A ratios by excluding the genes on the pseudo-autosomal region.
The bulk RNA-seq/SC3-seq datasets were re-analyzed by excluding genes on the pseudo-autosomal region. (A) The Chr.3:A and X:A ratios in mouse fetal/neonatal gonadal cells analyzed by bulk RNA-seq. Dots, individual data points; bar, mean. Samples at E9.5/10.5 include germ cells from male and female embryos (mix). E, embryonic day; P, postnatal day; Soma, gonadal somatic cells. (B) The Chr.15:A and X:A ratios in cy female germ cells *in vivo* and in cultured/transplanted rOvaries analyzed by SC3-seq. The Chr.15:A/X:A ratio transitions in the embryonic and hypoblast lineages reported by Okamoto *et al*. (2021) (Okamoto, Nakamura et al., 2021) are also shown. ESC, embryonic stem cell; ICM, inner cell mass; Pre_EPI, pre-implantation epiblast; PostE_EPI, post-implantation early epiblast; PostL_EPI, post-implantation late epiblast; Gast, gastrulating cells; ePGC, early primordial germ cells. Data with a small sample size (n≦3) are indicated with dots (individual data points) and bars (mean). (C) The Chr.10:A and X:A ratios in human fetal oocytes *in vivo* and in cultured rOvaries analyzed by SC3-seq.

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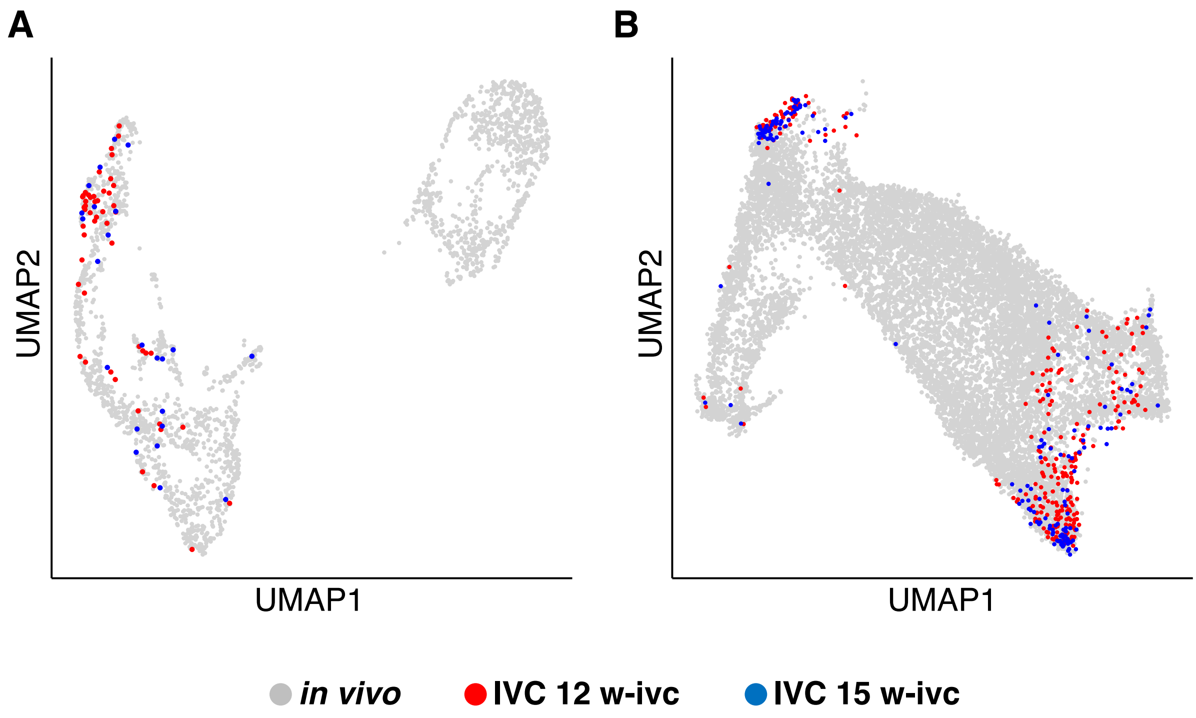
Mizuta et al., Figure R1

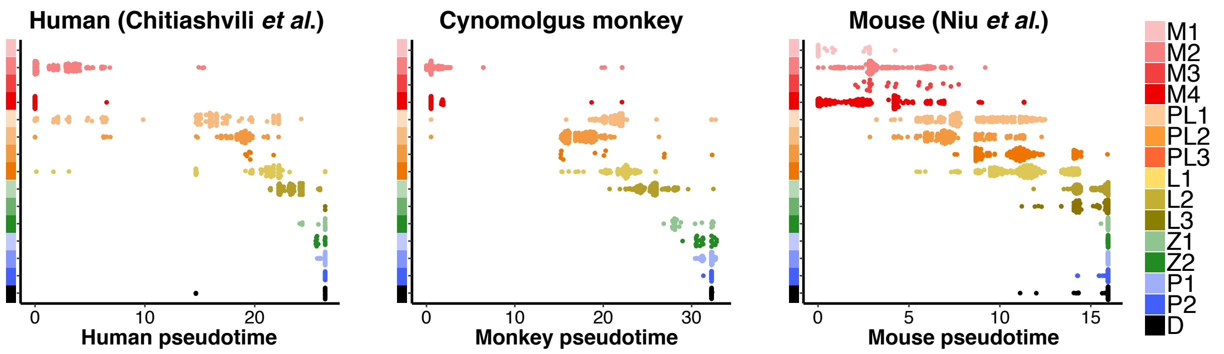


Mizuta et al., Figure R2

A

Germ-cell stage (%)		Human Chitiashvili *et al*.	Monkey	Mouse Niu *et al*.
Mitosis	(28.1%)	171	286	540
Pre-leptotene	(32.1%)	196	327	617
Leptotene	(23.3%)	142	237	447
Zygotene	(4.2%)	26	43	82
Pachytene	(10.0%)	61	102	192
Diplotene	(2.3%)	14	23	43
Total		610	1018	1921

B



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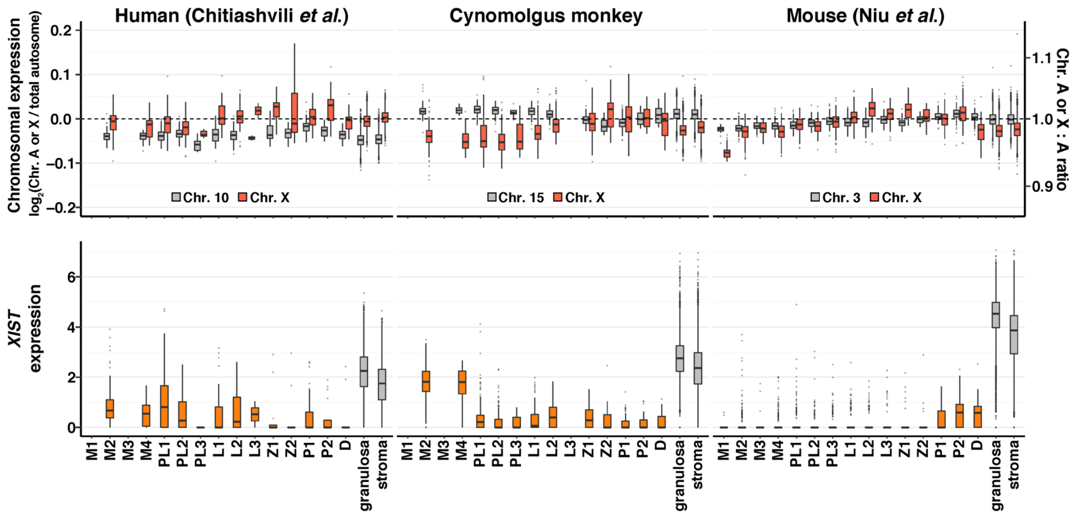
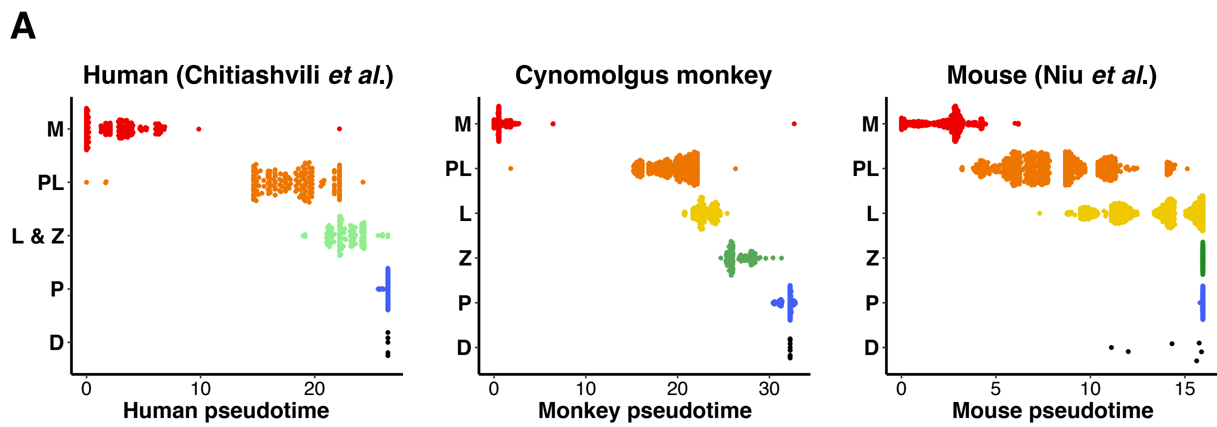
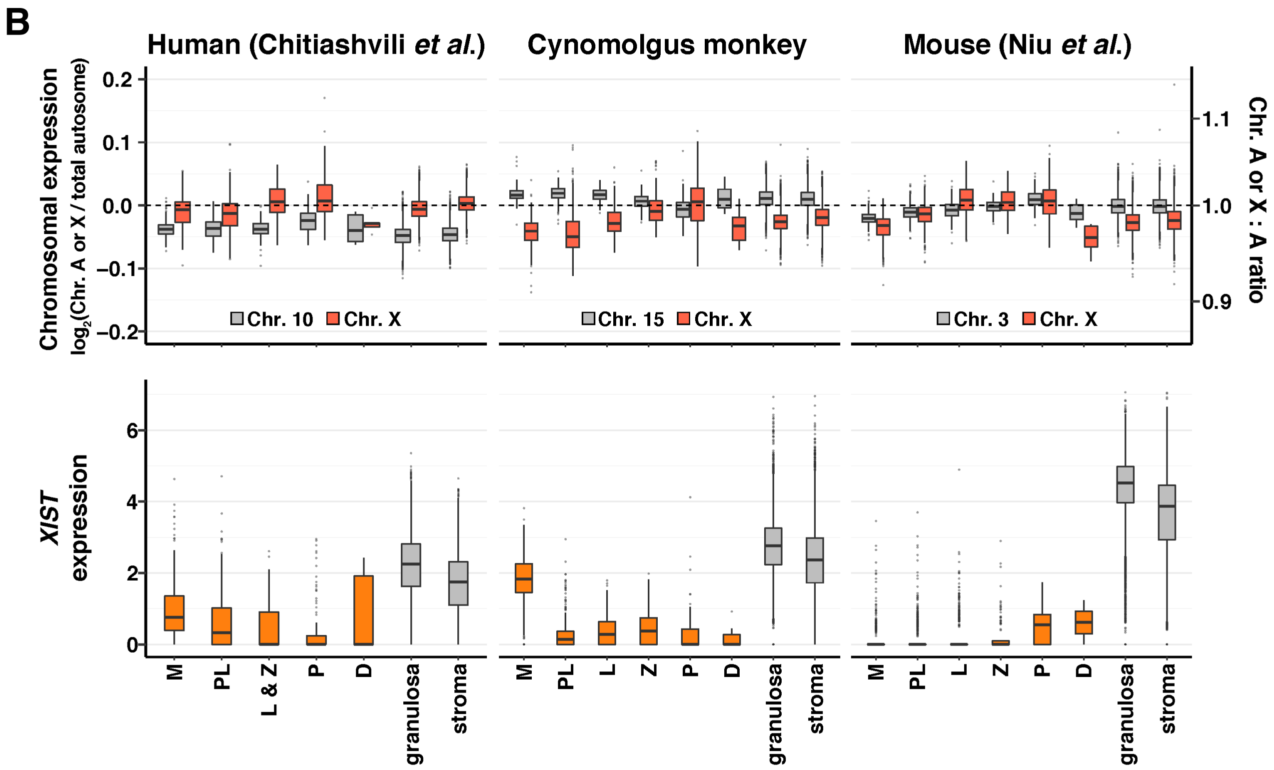
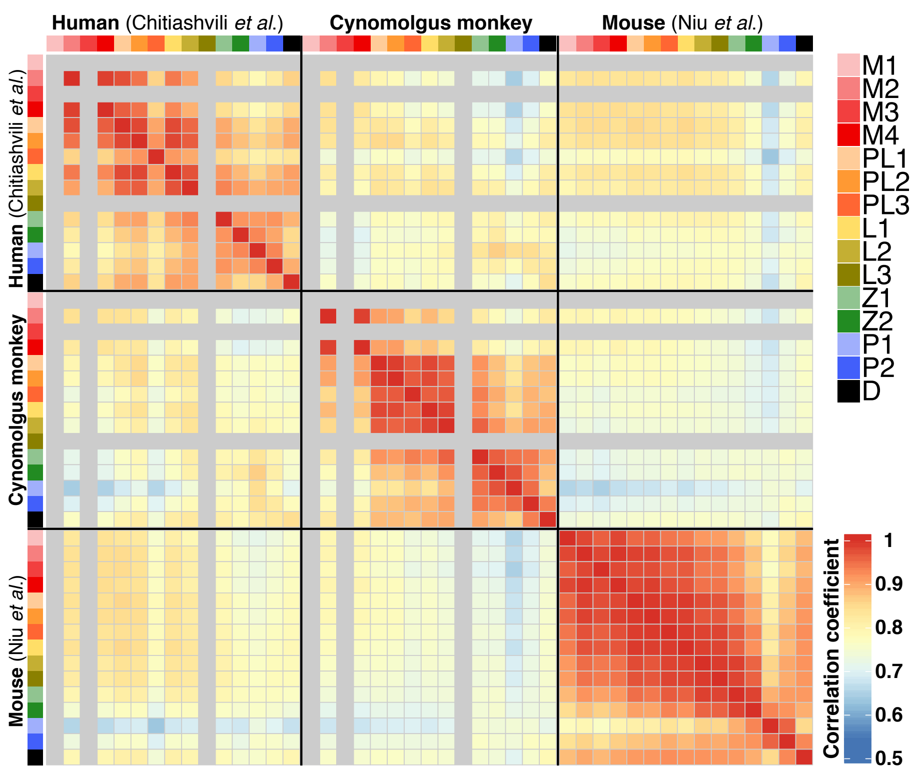


Mizuta et al., Figure R3





Mizuta et al., Figure R4

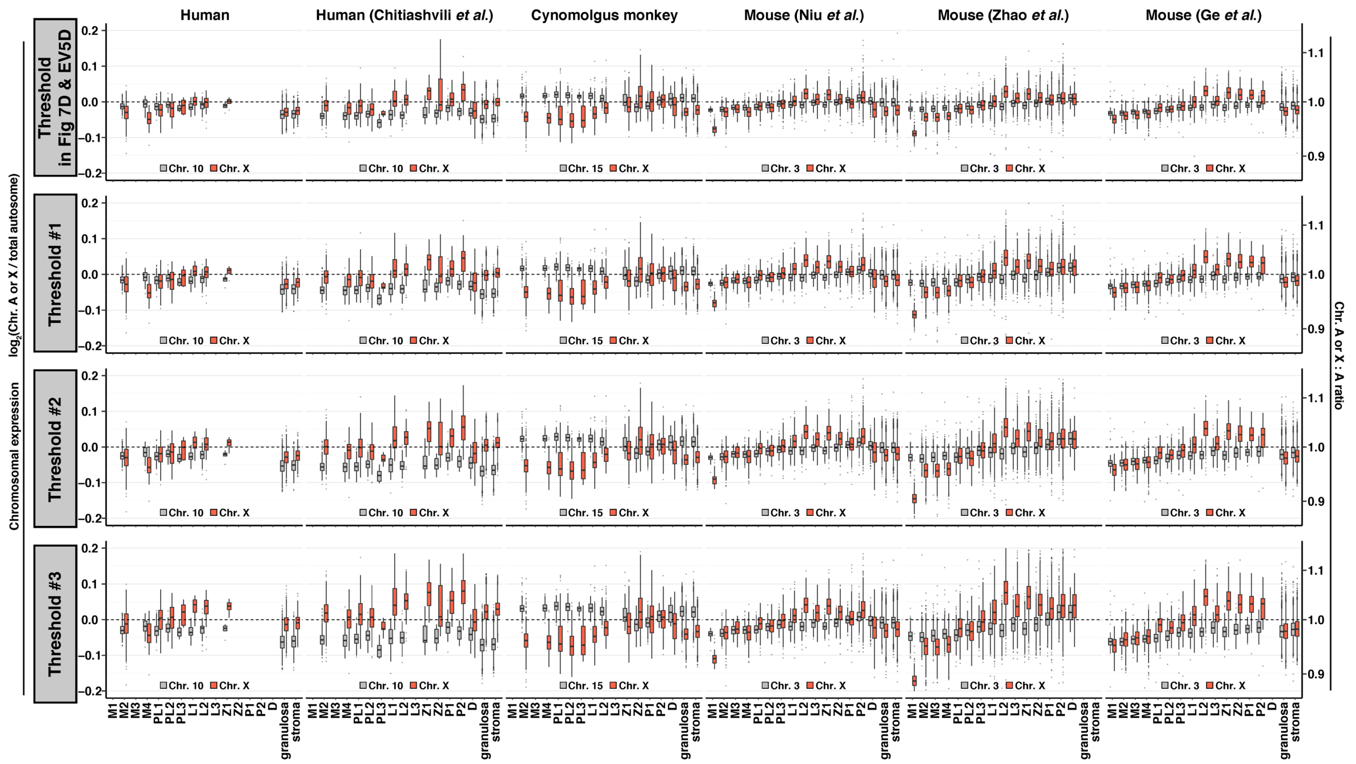


Mizuta et al., Figure R5

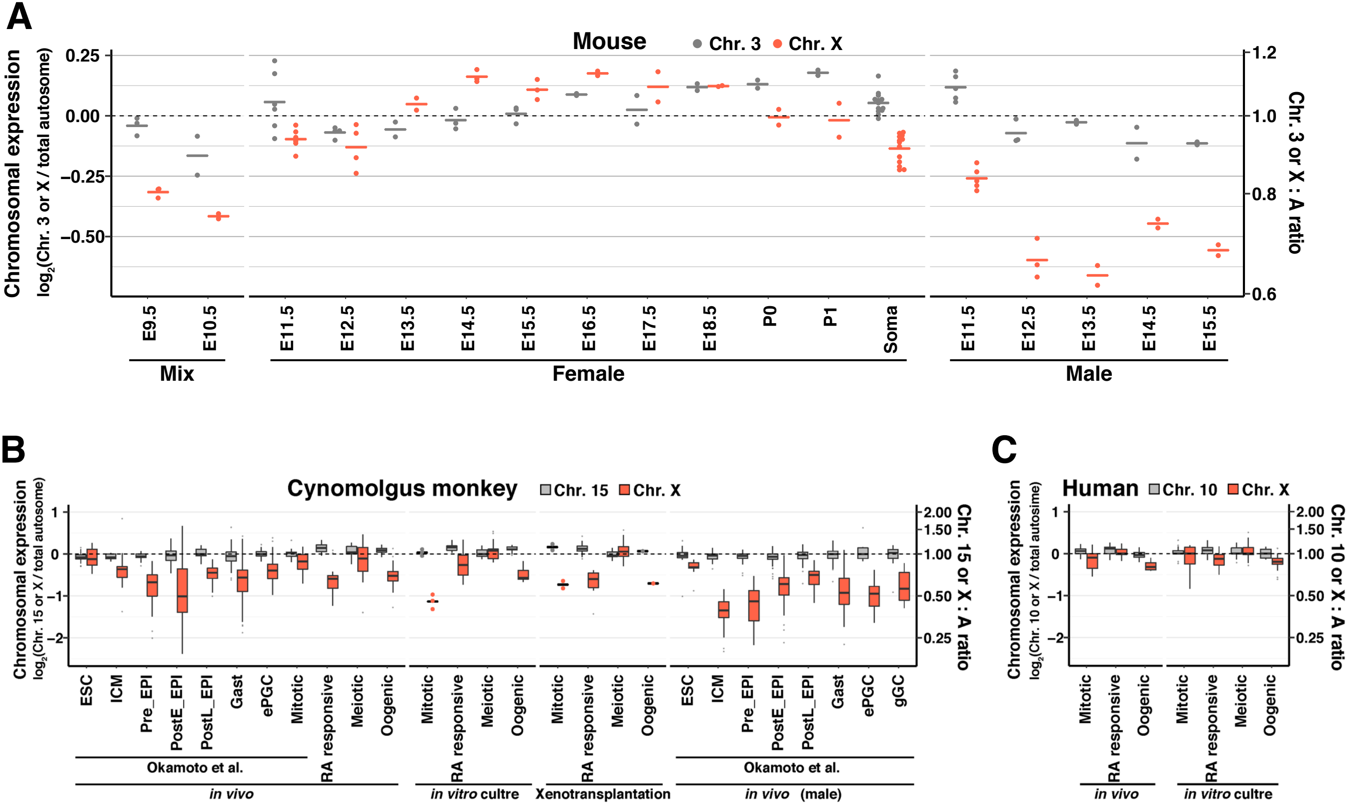
A

		Human	Human Chitiashvili *et al*.	Monkey	Mouse Niu *et al*.	Mouse Zhao *et al*.	Mouse Ge *et al*.
No threshold (i.e., all genes)	chr. X	858	858	839	961	961	961
	chr. A	739	739	784	985	985	985
	all autosomes	19,095	19,095	19,175	20,633	20,633	20,633
【Threshold in Fig 7D & EV5D】 $\log_2$(ss10kUMI+1) > 0 in more than 2 cells	chr. X	716	725	791	744	687	697
	chr. A	667	682	736	896	808	847
	all autosomes	16,862	17,190	18,066	17,698	16,545	16,821
【Threshold #1】 $\log_2$(ss10kUMI+1) > $\underline{1}$ in more than 2 cells, without genes on the PAR	chr. X	587	587	701	618	505	557
	chr. A	573	574	655	777	602	689
	all autosomes	14,443	14,585	15,972	15,357	12,441	13,931
【Threshold #2】 $\log_2$(ss10kUMI+1) > $\underline{\underline{2}}$ in more than 2 cells, without genes on the PAR	chr. X	512	497	625	546	399	470
	chr. A	511	507	581	685	473	584
	all autosomes	12,672	12,661	14,349	13,527	9,755	11,595
【Threshold #3】 $\log_2$(ss10kUMI+1) > $\underline{\underline{3}}$ in more than 2 cells, without genes on the PAR	chr. X	421	403	570	481	307	371
	chr. A	443	416	520	606	375	476
	all autosomes	10,962	10,747	13,040	11,712	7,625	9,258

B



Mizuta et al., Figure R6



1st Revision - Editorial Decision 24th Jun 2022

Dear Mitinori,

Thank you for submitting your revised manuscript (EMBOJ-2022-110815R) to The EMBO Journal. Your amended study was sent back to the three referees for their reassessment, and we have received comments from all of them, which I enclose below. As you will see, the experts stated that their issues have been comprehensively resolved and they are now broadly in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal.

We need you to take care of a number of minor issues related to formatting and data presentation as detailed below, which should be addressed at resubmission.

Please contact me at any time if you have additional questions related to below points.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision. to the swiftly proceed with acceptance of your article.

Again, please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

****

Formatting changes required for the revised version of the manuscript:

>> Please adjust the title of the 'Conflict of Interests' section to 'Disclosure and Competing Interests Statement'.

>> Data accessibility section: add an http link for the GEO dataset and release privacy at the acceptance date.

>> Author Contributions: specify contributions of M.Nak. versus M.Nag. in the Credit list.

>> Dataset EV legends: Tables EV1-EV8 & EV10 should all be renamed as Dataset EV#. Table EV9 should be changed to 'Table EV1'. The legends should be removed from the manuscript file.

>> Manuscript order: please move Materials & Methods after the Discussion section.

>> Indicate redisplay of Fig6E <'Mouse (Niu et al)' vs 'Mouse (Niu et al)'> part in the legend of Figure S8B.

>> Revise and amend figure callouts and their order. Fig EV2E callout is missing. Appendix Fig S4 is called out after S6. Appendix Fig S8A&B are called out before Appendix Fig S7C.

>> The Reagents and Tools table should be removed from the manuscript file, as it is already uploaded separately as a Reagents Table.

>> Please consider additional changes and comments from our production team as indicated by the .doc file enclosed and leave changes in track mode.

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Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

Please use the link below to submit your revision:

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Referee #1:

The authors have successfully addressed all of my comments. I support the publication of this manuscript in The EMBO journal.

Referee #2:

The authors have satisfactorily addressed my comments.

Referee #3:
This manuscript from the Saitou's laboratory is of high importance for the scientific community. I am satisfied with the answers to my review as well as to the two other reviewers.
I am supporting this manuscript for publication in EMBO Journal.

Rebuttal: EMBOJ-2022-110815R

Reviewers' comments

Reviewer #1:

The authors have successfully addressed all of my comments. I support the publication of this manuscript in The EMBO journal.

Reviewer #2:

The authors have satisfactorily addressed my comments.

Reviewer #3:

This manuscript from the Saitou's laboratory is of high importance for the scientific community. I am satisfied with the answers to my review as well as to the two other reviewers. I am supporting this manuscript for publication in EMBO Journal.

Responses to the Reviewers:

We would like to sincerely thank the Reviewers for their approval and encouraging comments on our manuscript.

2nd Revision - Editorial Decision 29th Jun 2022

Dear Mitinori,

Thank you for submitting the revised version of your manuscript EMBOJ-2022-110815R1. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

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Corresponding Author Name: Hiroshi Ohta, Mitinori Saitou
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2022-110815

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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.

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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.

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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.
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