Residual Pluripotency is required for Inductive Germ Cell Segregation

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Dear Prof. Schöler,

Thank you for transferring your manuscript to EMBO reports. I now went through your manuscript and the referee reports from The EMBO Journal (attached below). The referees acknowledge that the findings are of interest. Nevertheless, they have raised a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn.

EMBO reports emphasizes novel functional over detailed mechanistic insight, but asks for clear physiological relevance of the findings, and strong experimental support of the major conclusions. Thus, we will not require addressing points regarding more mechanistic details experimentally. However, it will be necessary that in a revised manuscript you address all points questioning the main conclusions of the study, and all technical concerns, or points regarding the experimental design, model systems used, or data presentation. In particular, it would be important to address the concerns regarding novelty (referee #1) and physical relevance (e.g. point 3 of referee #1 and point 1 of ref. #2).

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript and/or in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

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The accession numbers and database should be listed in a formal "Data Availability " section (placed after Materials & Methods) that follows the model below. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

# Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843
  (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
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Moreover, I have these editorial requests:

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.
Referee #1:

This paper explores the combined roles of the mesodermal transcription factor, T, and pluripotency factors, particularly Oct4, in inducing germ cell fate from naïve ES cells. They show that expression of high levels of ectopic T repress pluripotency and promote somatic differentiation, but that lower levels of T result in residual expression of Oct4 and other pluripotency factors and the induction of germ cell markers like Blimp1, Stella and Prdm14. They show by Chip-Seq that T binds to specific regions around some of the germ cell determinant genes in association with Oct4. These co-bound regions were activated by expression of T, but activation was lost upon deletion of Oct4.

The data as presented are quite convincing in suggesting a role for T in directly activating determinants of the germ cell lineage in naïve ES cells, and that this activation is dependent on the continued expression of pluripotency genes. Given that all the data are produced by ectopic expression of T in ES cells, it is not quite so clear how this all relates to the events of germ cell determination in the embryo itself. Nor is it clear how these results suggesting direct induction of the germ cell lineage from naïve ES cells relate to other studies showing that naïve cells need to enter an EpiLC state before germ cell induction. They mention this in the discussion but do not fully provide a unifying hypothesis.

Questions to address:

1. They say in the introduction that it is not known how T acts to specify the germ cell lineage, and yet they refer to the paper by Aramaki et al which has a very detailed analysis of how T acts to specify germ cells from EpiLCs. BMP4/Wnt signals through b-catenin to activate T, which then binds to sites associated with Blimp1 and Prdm14 to activate their expression. Aramaki et al did not look at the co-expression and binding of pluripotency factors, but it should be easy to compare the Chip-Seq data in the two papers to see if the key T binding sites are the same in the two studies. The current study does not address any possible upstream role for the known signaling pathways involved in germ cell induction. Are they thinking that ectopic expression of T in naïve cells basically allows skipping of the intermediate Wnt/BMP4/b-catenin activation stage?
2. The finding that the induction of germ cell fate by ectopic T only occurs when levels are low enough not to fully block pluripotency and promote somatic differentiation is clearly presented. However, it is not so clear exactly what is meant at a quantitative level in terms of both gene expression and DNA binding activity by high, medium or low T activity. Is this response a continuum or are there defined thresholds of action? What is the actual state of occupancy of the T/Oct4 binding sites associated with germ cell determinant genes at different levels of T expression?
3. Is the in vivo region of germ cell induction at the posterior primitive streak consistent with the idea that low levels of T promote germ cell development? What is the level of expression of T and Oct4 in the germ cell domain? If levels of T do not vary along the streak, is there actually cross-talk with the BMP4 domain of action, which is confined to the posterior primitive streak?
4. T binds to enhancer regions of Oct4 and represses expression to disrupt pluripotency. How does this repression work? Is T a direct repressor or does it block activators of Oct4 expression?
5. T can repress germ cell determinant genes at high levels and promote expression at low levels. The ChipSeq data for T is presumably derived from high level expressing lines, where T does not activate expression. Does this mean that T is always bound to these genes whether or not Oct4 is present? Is high T actually involved in repression itself, or is this a secondary consequence of losing Oct4 cobinding?

Referee #2:

In this manuscript, the authors reported a novel function of the mesoderm-related transcription factor T/Brachyury on the exit of pluripotency and the commitment to the germ cell fate. They previously reported that the expression levels of T in the primed pluripotent stem cells, mouse epiblast stem cells (EpiSCs), show the relationship to the reprogramming efficiency toward the naïve state (Bernemann et al, 2011). Here they investigated the molecular mechanism of the action of T to counteract the pluripotency-associated transcription factors using the inducible expression of T in EpiSCs and embryonic stem (ES) cells. They found that the induction of T interfere with the reversion of EpiSCs to the naïve state and the chimera contribution of all pluripotent stem cell lines. The inhibitory function of T on the maintenance of pluripotency show dose-dependent effect in which the low dose triggers induction of the germ cell-related genes with keeping the core pluripotency factors whereas the high dose induces the mesoderm-related genes with suppression of pluripotency genes. Mechanistically, T co-occupies the genomic regions with pluripotency-associated transcription factors such as Pou5f1 and inhibit their transcriptional activation. T is famous for its role in mesoderm differentiation. However, it was reported that T is essential for induction of germ cells in mouse embryos and in vitro differentiation of mouse ES cells (Aramaki et al, 2013). The molecular mechanisms to mediate the different functions of the single transcription factor in distinct developmental context is important but has not been well characterized. Here the authors demonstrated the dose-dependent action of T to induce the germ-cell-related genes. This is a novel finding and worth to be published in EMBO J. However, there are several points that should be addressed in the revised manuscript.

1. The authors applied the inducible expression system in cultured cells to achieve the ectopic expression of T, but there is no information about its expression level in comparison to the endogenous expression levels in different developmental context. Is the expression level in 'high' population in the physiological range? The precise estimation of the expression level should be addressed in the revised manuscript.
2. How about the relationship between the expression levels of core pluripotency factors (Pou5f1), naïve-specific factors (Klf4) and T in embryos at different developmental stages? Are there any heterogeneity in the epiblast cells of early post-implantation embryos?
3. In Fig 2B, the blot with longer exposure time of KLF4 should be presented.
4. The molecular mechanism to mediate down-regulation of naïve-specific factors such as Klf4 at low T expression is not well characterized. The reporter assay in Fig 3B showed that all reporters are down-regulated in T-low cells. How is the selective down-regulation achieved?
5. In Fig 5, the authors demonstrated that the low level expression of T triggers PGC-like transcriptional character. Do the T-induced PGCLCs undergo differentiation to mature germ cells?
6. There is no explanation and citation for ZHBTC4 ES cells.
We would like to sincerely thank the referees for their encouraging and constructive comments, which have been instrumental in our revision of the manuscript.

Referee comments:
Referee #1:

This paper explores the combined roles of the mesodermal transcription factor, T, and pluripotency factors, particularly Oct4, in inducing germ cell fate from naïve ES cells. They show that expression of high levels of ectopic T repress pluripotency and promote somatic differentiation, but that lower levels of T result in residual expression of Oct4 and other pluripotency factors and the induction of germ cell markers like Blimp1, Stella and Prdm14. They show by Chip-Seq that T binds to specific regions around some of the germ cell determinant genes in association with Oct4. These co-bound regions were activated by expression of T, but activation was lost upon deletion of Oct4.

The data as presented are quite convincing in suggesting a role for T in directly activating determinants of the germ cell lineage in naïve ES cells, and that this activation is dependent on the continued expression of pluripotency genes. Given that all the data are produced by ectopic expression of T in ES cells, it is not quite so clear how this all relates to the events of germ cell determination in the embryo itself. Nor is it clear how these results suggesting direct induction of the germ cell lineage from naïve ES cells relate to other studies showing that naïve cells need to enter an EpiL state before germ cell induction. They mention this in the discussion but do not fully provide a unifying hypothesis.

Response 1. We would like to sincerely thank the Referee for his/her acknowledgement of the highlight in the study and pointing out the issues to be clarified, especially about the physiological relevance and the direct induction of germ cell fate from naïve pluripotency. In the revised manuscript, we addressed these major issues to improve our manuscript.

Questions to address:

1. They say in the introduction that it is not known how T acts to specify the germ cell lineage, and yet they refer to the paper by Aramaki et al which has a very detailed analysis of how T acts to specify germ cells from EpiLCs. BMP4/Wnt signals through b-catenin to activate T, which then binds to sites associated with Blimp1 and Prdm14 to activate their expression. Aramaki et al did not look at the co-expression and binding of pluripotency factors, but it should...
be easy to compare the Chip-Seq data in the two papers to see if the key T binding sites are the same in the two studies. The current study does not address any possible upstream role for the known signaling pathways involved in germ cell induction. Are they thinking that ectopic expression of T in naïve cells basically allows skipping of the intermediate Wnt/BMP4/B-catenin activation stage?

Response 2. In accordance with the Referee’s correct suggestion, we examined in more detail how direct the T-dependent induction of germ cell fate from the naïve state is or how it relates to the previously identified upstream signaling pathways required for PGC specification. As shown in Fig EV4B and C in the revised manuscript, the activation of germ cell program was induced as early as 8 hours after T induction in 2iLIF ESCs with faster kinetics than the previous PGCLC induction through EpiLC-derivation for two days and the following cytokine stimulation for at least 3-4 days. Importantly, this activation of the germ cell fate program was not affected by the addition of chemical inhibitors of BMP and WNT signaling pathways (LDN193189 and XAV939, respectively), or withdrawal of ChIR99201 (activator of WNT/B-CATENIN pathway). In addition, the activation was induced in the context of repressed target genes (ldl and Axin2) downstream of BMP and WNT signaling pathways, respectively (Fig EV4C and Appendix Fig S4A in the revised manuscript). We thus consider that these additional findings strongly suggest that our induction model is independent of the transition to the primed state like EpiLCs and the activation of signaling pathways required for germ cell fate induction. We have included a relevant passage in the revised manuscript (Lines 210-222 of text).

In terms of the relationship with the previous study, as the Referee comments, we compared the T ChIP-seq data of this study and the previous analysis (Aramaki et al., 2013) and found that some of T binding peaks in germ cell determinants loci are well matched between two independent studies (Appendix Fig S6, Lines 333-336 of text). At the same time, we would like to point out to the Referee that we identified this pathway in the process of investigating the disruption pathway of naïve pluripotency by T. This actually led us to the focus on the coregulatory mechanism through T and pluripotency factors for germ cell fate induction, the following comparative analysis of T (this study) and core pluripotency factors in the naïve state (Adachi et al., 2017), and the consequent hypothesis supported by functional analysis (Fig 5).

2. The finding that the induction of germ cell fate by ectopic T only occurs when levels are low enough not to fully block pluripotency and promote somatic differentiation is clearly presented. However, it is not so clear exactly what is meant at a quantitative level in terms of both gene expression and DNA binding activity by high, medium or low T activity. Is this response a continuum or are there defined thresholds of action? What is the actual state of occupancy of the T/Oct4 binding sites associated with germ cell determinant genes at different levels of T expression?
Response 3. According to the Referee’s comment, we examined the actual localization of T to the co-bound loci of Blimp1 and Prdm14 in respective dose fractions (low, middle, and high T) by ChIP-qPCR. This analysis clearly revealed that T-binding to these loci increased in a dose dependent manner, indicating that PGC determinants were repressed despite the binding of T to the regulatory loci. This is also consistent with our notion that activation of these germ cell fate determinants is dependent on other regulatory factors like pluripotency activators. We have included these data and a corresponding passage in the revised manuscript (Fig EV5D, Lines 260-264 of text).

3. Is the in vivo region of germ cell induction at the posterior primitive streak consistent with the idea that low levels of T promote germ cell development? What is the level of expression of T and Oct4 in the germ cell domain? If levels of T do not vary along the streak, is there actually cross-talk with the BMP4 domain of action, which is confined to the posterior primitive streak?

Response 4. In accordance with the Referee’s suggestion, we examined whether our in vitro finding were physiologically equivalent to the in vivo context. To evaluate the level of T at early germ cell specification stages (E7.0-7.5) in vivo, we utilized T-GFP knock-in reporter system. Observation by confocal microscopy and the FACS analysis revealed that initially induced Blimp1-positive PGCs were detected in the region of low T levels in the posterior proximal epiblast (Fig 4E and F, Fig EV4F, Lines 238-247 of text). In addition, qPCR analysis revealed that pluripotency factors such as Oct4 were relatively well preserved in Blimp1-positive nascent germ cells with low T levels compared with the cell population expressing high T levels. On the other hand, we also found that the majority of T-low cell population had no or lower Blimp1 expression as this Referee correctly assumed. Regarding this issue, we showed that the Id1 gene (inhibitor of differentiation/DNA binding 1, an activator of in vitro naïve pluripotency (Ying et al., 2003; Romero-Lanman et al., 2011)), a downstream target of BMP/SMAD signaling, was more activated in Blimp1-positive, T-low-positive PGCs compared with Blimp1-negative/low-positive, T-low-positive cells. This suggests that cross-talk with BMP signaling is also critical for germ cell induction in vivo. We have incorporated these data and a corresponding passage into the revised manuscript (Fig EV4F, Lines 313-322 of text).

4. T binds to enhancer regions of Oct4 and represses expression to disrupt pluripotency. How does this repression work? Is T a direct repressor or does it block activators of Oct4 expression?

Response 5. Many previous studies using mouse ESCs and several other organisms have shown that T functions as a transcriptional activator but not a repressor (Reviewed in Showell et al., 2004; Lolas et al. 2013), whereas on the other hand, our data clearly indicate that T represses pluripotency factors such as Oct4. In addition, our T-ChIP-seq revealed that induced T is localized in regulatory elements such as the Oct4 distal enhancer locus, where many
pluripotency activators are colocalized in advance (Fig 3). Thus, we examined whether T affects the binding of preexisting pluripotency regulators localized on the enhancer loci that are also the target sites of induced T, leading to the resulting repressor-like behavior in naïve ESCs.

We examined this possibility representatively at the Oct4 distal enhancer locus (Fig EV3G in the revised manuscript) and found that binding of core pluripotency factors such as OCT4 to the Oct4 distal enhancer locus was inhibited in T dose dependent manner already at 3 hours after T induction, whereas expression of these core factors was still unaffected at this early time point, as shown by FACS and qPCR analysis. These data suggest that in the naïve pluripotency context where transcriptional activation of pluripotency genes is already highly activated (or presumably saturated), the interference effect of T can be more dominant than its activation effect. We incorporated a relevant passage into the revised manuscript (Lines 144-157 of text).

5. T can repress germ cell determinant genes at high levels and promote expression at low levels. The ChipSeq data for T is presumably derived from high level expressing lines, where T does not activate expression. Does this mean that T is always bound to these genes whether or not Oct4 is present? Is high T actually involved in repression itself, or is this a secondary consequence of losing Oct4 cobinding?

Response 6. As this Referee correctly surmises, T is localized in the germ cell-determining genes at all the examined dose levels (Please also see Response 2 and the related Fig EV5D). Even in the high T fraction, where germ cell genes and pluripotency factors such as Oct4 are markedly repressed (Fig 1-3), T is localized in such germ cell gene loci, suggesting that T-binding to germ cell genes is independent of the pluripotency state. On the other hand, as we have described in Response 4, many previous studies have shown that T itself functions as a transcriptional activator, and our additional experiment suggests that T can behave like a transcriptional repressor in the naïve state by interfering with the binding of pluripotency activators like OCT4 to regulatory elements (Fig EV3G). Based on these findings, we think it is logical to assume that the downregulation of germ cell genes is secondary consequence of pluripotency disruption rather than a direct repressive effect by T. We also think that this notion is supported by the data in the first manuscript, showing that low T-dependent induction of germ cell genes and the transcriptional activation of their specific enhancers are markedly repressed by Oct4 deletion (Fig 4A, Fig EV5A, Fig 5C). We incorporated a passage related with this point into the revised manuscript (Lines 260-264 of text).

Referee #2:

In this manuscript, the authors reported a novel function of the mesoderm-related transcription
factor T/Brachyury on the exit of pluripotency and the commitment to the germ cell fate. They previously reported that the expression levels of T in the primed pluripotent stem cells, mouse epiblast stem cells (EpiSCs), show the relationship to the reprogramming efficiency toward the naïve state (Bernemann et al, 2011). Here they investigated the molecular mechanism of the action of T to counteract the pluripotency-associated transcription factors using the inducible expression of T in EpiSCs and embryonic stem (ES) cells. They found that the induction of T interferes with the reversion of EpiSCs to the naïve state and the chimera contribution of all pluripotent stem cell lines. The inhibitory function of T on the maintenance of pluripotency show dose-dependent effect in which the low dose triggers induction of the germ cell-related genes with keeping the core pluripotency factors whereas the high dose induces the mesoderm-related genes with suppression of pluripotency genes. Mechanistically, T co-occupies the genomic regions with pluripotency-associated transcription factors such as Pou5f1 and inhibit their transcriptional activation.

T is famous for its role in mesoderm differentiation. However, it was reported that T is essential for induction of germ cells in mouse embryos and in vitro differentiation of mouse ES cells (Aramaki et al, 2013). The molecular mechanisms to mediate the different functions of the single transcription factor in distinct developmental context is important but has not been well characterized. Here the authors demonstrated the dose-dependent action of T to induce the germ-cell-related genes. This is a novel finding and worth to be published in EMBO J. However, there are several points that should be addressed in the revised manuscript.

Response 1. We would like to sincerely thank the Referee for his/her encouraging comments on our manuscript.

1. The authors applied the inducible expression system in cultured cells to achieve the ectopic expression of T, but there is no information about its expression level in comparison to the endogenous expression levels in different developmental context. Is the expression level in 'high' population in the physiological range? The precise estimation of the expression level should be addressed in the revised manuscript.

Response 2. According to the referee’s comment, we examined whether the induced T in vitro is at physiological level by comparing it with in vivo T expression in the cell population of higher T level defined by FACS. Western analysis revealed that T expression of high T fraction in vitro was comparable to that of the high T fraction in vivo, with no significant difference between the high T fractions in vitro and in vivo, suggesting that the induced T in our experimental model in vitro is in the physiological level. We incorporated these data and a relevant passage into the revised manuscript (Fig 4E, Fig EV4E, Lines 238-244 of text).

2. How about the relationship between the expression levels of core pluripotency factors
(Pou5f1), naïve-specific factors (Klf4) and T in embryos at different developmental stages? Are there any heterogeneity in the epiblast cells of early post-implantation embryos?

Response 3. In accordance with the Referee’s suggestion, we evaluated the in vivo expression level of T and pluripotency factors at two different post-implantation/germ cell specification stages utilizing T-GFP and Blimp1-RFP double reporter system. We found that in vivo, pluripotency factors such as Oct4 and Klf4 are also relatively downregulated in the high T cell population compared with the T low cell population such as PGCs. We incorporated this data and a relevant passage into the revised manuscript (Fig 4E and F, Fig EV4F, Lines 238-247 of text).

3. In Fig 2B, the blot with longer exposure time of KLF4 should be presented.

Response 4. We provided the western data of KLF4 with longer exposure time in Fig 2B in the revised manuscript.

4. The molecular mechanism to mediate down-regulation of naïve-specific factors such as Klf4 at low T expression is not well characterized. The reporter assay in Fig 3B showed that all reporters are down-regulated in T-low cells. How is the selective down-regulation achieved?

Response 5. We consider that each transcriptional activation value measured in the luciferase assay originates from only one specific regulatory site, and thus does not always correspond to the level of change in endogenous gene expression, which may be a cumulative consequence influenced by other factors such as other regulatory elements and related transcription factors. Indeed, there are small binding peaks (putative regulatory loci) around the pluripotency gene loci examined (Fig 3A and Fig EV3E). We described this notion in the revised manuscript (Lines 136-143 of text).

5. In Fig 5, the authors demonstrated that the low level expression of T triggers PGC-like transcriptional character. Do the T-induced PGCLCs undergo differentiation to mature germ cells?

Response 6. According to the referee’s comment, we examined whether the T-induced PGCLCs (T-PGCLCs) proceed to further germ cell differentiation by using the previously established method (Hikabe et al., 2016). However, we found that the efficiency of derivating primary oocytes from T-PGCLCs was much lower than that of in vivo PGCs. We hypothesize that this is due to incomplete specification of T-PGCLCs presumably due to the continual activation of T. Indeed, previous studies have shown that mesodermal genes, including T, are eventually repressed in PGCs and continued activation of the somatic mesodermal program is detrimental.
to the induction of germ cell fate (Ohinata et al., 2005; Kurimoto et al., 2008). We incorporated this data and a corresponding passage into the revised manuscript (Appendix Fig S4D, Lines 232-237 of text, Lines 305-311 of text).

6. There is no explanation and citation for ZHBTC4 ES cells.

Response 7. We thank the Referee for pointing this out. We have incorporated the proper reference into the revised manuscript (Lines 165 of text).

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Ying QL, Nichols J, Chambers I, Smith A (2003) BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell 115: 281-292
Dear Prof. Schöler,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the two referees that were asked to re-evaluate your study, you will find below. As you will see, both referees now fully support the publication of your study in EMBO reports.

Before we can proceed with formal acceptance, I have these editorial requests I ask you to address in a final revised version of the manuscript:

- Please add your funding information into the online submission system and make sure that it is complete and similar to the one in the manuscript (in the Acknowledgements).

- The Tables EV1, EV2 and EV3 are too large to be displayed as tables in the online version of the manuscript. These should be datasets. Please name and upload these as Dataset EV1/2/3 and add their legend on the first TAB of the excel file. Please remove then their legend from the manuscript and update the callouts in the manuscript text.

- The present EV Tables 4 and 5 are fine, but these should then be renamed EV1 and EV2, respectively. Please also put their legend on the first TASB of the excel file, remove the legend from the text and update the callouts in the manuscript text.

- For the microscopic images, please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

- In the Appendix file, please move the legends below the respective figures. I think this is easier for the readers to comprehend. Could you also provide a title (Appendix for 'Residual Pluripotency is required ...') and remove the word 'supplementary' from the Appendix TOC.

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also of the EV and Appendix figures), and that statistical testing has been done where applicable.

- As the Western blots shown are significantly cropped, could you provide the source data for all the blots (main and EV figures). The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. Please submit the source data (scans of entire blots) together with the revised manuscript. Please include size markers for scans of entire blots, label the scans with figure and panel number and send one PDF file per figure.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see any modifications done.
In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four bullet points highlighting the key findings of your study.
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Kind regards,

Achim Breiling
Editor
EMBO Reports

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Referee #1:

In this revised manuscript, the authors added several new data to answer the questions by the reviewers. This reviewer is now satisfied by their appropriate revisions.

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Referee #2:

The authors have responded with new experiments to the questions raised by the reviewers. These new data have strengthened their case in terms of the in vivo relevance of the levels of T for germ cell induction. They also show that ectopic T expression alone is not sufficient to generate fully functional germ cell precursors and that this probably implicates an interaction with the endogenous BMP signaling pathway. This makes some sense in terms of the in vivo production of germ cells in the embryo as well as helping define the components important for germ cell induction from ES cells in culture. This is an interesting new angle on the germ cell induction pathway.
The authors have addressed all minor editorial requests.
Dear Prof. Schöler,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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Yours sincerely,

Achim Breiling
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| 2. Captions | Each figure caption should contain the following information, for each panel where they are relevant: |
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| 1. Data | The data shown in figures should satisfy the following conditions: |
| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | Sample size was decided based on previous studies in the field and experience with the variance of the measured data. Basically, each of the experimental group has at least two biological replicates. Due to ethical issue, the minimum number of animals required for the scientific objectives was used for animal work. |
| 1.b. For animal studies, include a statement about randomization even if no randomization was used. | The randomization procedure was taken for some animal experiments. |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | In order to evaluate the chimeric contribution, some of chimeras were excluded because of due to the loss of some embryonic parts during the dissection. |
| 3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe. | The randomization procedure was taken for some animal experiments. |
| 4. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes, please describe. | Blinding of the investigator was done in the chimeric contribution assay. |
| 5. For animal studies, include a statement about blinding even if no blinding was done. | Genetic contribution analysis, e.g. a pool of mice was screened by two individuals and one of them didn’t know the introduction of the genotype of cell lines which was utilized in the analysis. |
| 6. For every figure, are statistical tests justified as appropriate? | Yes, for statistical analyses in this study, p-values were calculated by two-tailed unequal t-test. |
| 7. If the data meet the assumptions of the tests (e.g., normal distribution? Describe any methods used to assess it. | The data of mRNA expression showed normal distribution, which was checked by Shapiro-Wilk algorithm. |
| 8. Are there an estimate of variation within each group of data? | Yes. We presented the data as means ± standard deviation (SD). |
| 9. Is the variance across the groups that are being statistically compared? | Yes. We tested for significant differences between the groups using ANOVA and a post-hoc test (Tukey's) where appropriate. |

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D- Animal Models

8. Describe species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.

9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

10. We recommend consulting the ARRIVE guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under Reporting Guidelines. Please confirm you have submitted this list.

11. Procedures used in the present study followed the ethical and experimental recommendations of the German Society of Laboratory Animal Science. On the local regulatory level, mice were used for experiments according to the institutional approval issued by the Landesamt für Natur, Umwelt und Verbraucherschutz of the state of North Rhine-Westphalia, Germany (permit nos. 84-02.04.2016.A131, 85-01.04.2017-A376, and 85-03.04.2017-A405).

F- Data Accessibility

16. Report any restrictions on the availability (and/or on the use) of human data or samples.

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18. Provide a "Data Accessibility" section at the end of the Materials & Methods. Listing the accession codes for data generated in the study and deposited in a public database (e.g., RNA-seq data: Gene Expression Omnibus (GEO) GSE39662, Proteogenomics data: PROTEOCHIP10280 etc.) Please refer to our author guidelines for "Data Depositor".

19. Data deposition in a public repository is mandatory for:
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   b. Microarrays
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24. Report the clinical trial registration number (if ClinicalTrials.gov or equivalent), where applicable.

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27. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, each dataset should be deposited in one of the major public access/repositories such as dbGaP (see link list at top right) or Figshare (see link list at top right), as described in the "Data Availability" section at the end of the Materials & Methods.

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