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Human primordial germ cell commitment *in vitro* associates with a unique PRDM14 expression profile

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Editors: Alexander Kohlmaier (AK), Thomas Schwarz-Romond (TS-R), Bernd Pulverer (BP)

1st Editorial Decision (AK) 24 February 2014

Thank you for submitting your manuscript " PGC commitment in humans exhibits a unique PRDM14 expression pattern" to The EMBO Journal editorial office.

We have received the comments from three referees. They all express interest in principle, but raised a number of concerns that in particular relate to the conclusiveness of the submitted data:
Firstly, and beyond correlative evidence, reviewers #1 and #3 request functional (genetic?) evidence to confirm the hypothesis that PRDM14 is indeed not required for human germline development.
Secondly, all three reviewers point out that numbers and characteristics of PGC precursors in the studied system are not sufficiently addressed. As such, instead of forming a mesoderm-committed germ cell precursor, the relevant pool of cells might equally be rather heterogenous, with cells committed to either a mesoderm- or germline fate. Thus, interpretations from the analysis of FACS-sorted cells during the progressive differentiation from precursors to specified PGCLCs require additional and critical characterization/definition as well as precise quantifications.
We therefore kindly ask you to fully resolve these crucial issues, before being able to offer any further consideration at The EMBO Journal.

In addition, I like to stress that all other points raised by the three referees would need to be addressed experimentally in the course of a major revision. As these appear constructive and self-explanatory, there is no need to repeat them in detail here.
I am fully aware that these experimental additions are rather demanding. I am however certain that they would result in a dramatically improved and thus significantly stronger and informative study, as already indicated by some of the referees' encouraging remarks.
We would therefore be pleased if you would be willing to invest the necessary time and effort to accommodate this with additional time beyond our usual three months revision period, but we would at this point also understand if you were to decide to seek more rapid publication in an alternative journal.
I would be delighted if you were willing to pursue revisions for our title. Please also do not hesitate to potentially outline feasibility and anticipated timeline, in case you decide on this option.
We generally allow three months as standard revision time. In the light of the nature of the specifics of the experiments requested here, we would in principle be open to extend the deadline if necessary subject to considering the literature closer to the deadline.
Please note that it is our policy that competing manuscripts published during the revision period would have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

REFEREE COMMENTS

Referee #1:

The authors have provided a new approach for differentiating human germ cells from human pluripotent stem cells. The findings are in most cases solid and the hypothesis that Prdm14 is not associated with human germline development is provocative. The authors need to provide some functional evidence on the role (or not) of Prdm14 in human germline development.
Introduction:
It is hard to conceive how 10-15 weeks corresponds to mid to late stages of development in humans because gestation is 40 weeks, wouldn't it be early?
In the introduction the authors list a number of genes in common between mouse and human, but the reference to all these genes is a review. Can the authors use references from the primary literature for these.
The argument that PRDM14 represses NANOS3 and BMP4 is based upon work using mouse pluripotent cells. The phrasing of the argument suggests that the repression of NANOS3 by PRDM14 is a human phenomenon and is misleading.

Results:
The Activin and BMP induction protocol is very interesting and the increase in an intermediate cell between mesoderm and germline. However an alternate interpretation is that it could also mean a heterogeneous mix of mesoderm and germ line cells. Can the authors address heterogeneity?
Can the authors supply a better triple stain with T in Figure 1D. Also, can the authors provide quantification on the number of triple positive cells at day 2 based on number of cells plated.
The inability to sort GFP+ cells is concerning. Given the authors use an alternate approach for the remainder of their work, it doesn't make sense to include the STELLA-GFP reporter in Fig 1A or 2B or 4B and C bottom panel. Without sorting results, the specificity of this reporter cannot be appropriately validated.

Figure legend for 2D indicates that differentiation is shown up to day 10, however the last day indicated is day 8, is it day 10 or day 8.

The 5mC immunofluorescence stain in Figure 3D shows two different types if morphologies with iPS cells being spread out whereas the putative PGCs are bunched together making interpretation difficult. Can the authors show loss of 5mC by an alternative technique or different image where the representative cells between control and experiment are similarly placed relative to each other.

In the ovary experiment shown in Figure 3E, no teratoma formed when PGCs were used for transplantation, but what happened to the human cells? Did they survive but not proliferate, did the

e12.5 somatic cells form an ovarian-like structure or were all grafted cells cleared away? Can the authors provide more experimental details to facilitate interpretation of this experiment.

Figure 4C can the authors show higher power images or inserts.

The reduction in Dnmt1 levels in PGCs (Figure 5D) is surprising as in murine PGCs specification Dnmt1 RNA levels remain constant. Further, both Dnmt3a and Dnmt3b are reduced in mouse PGCs, whereas it appears only Dnmt3b is reduced in human in vitro PGCs with differentiation. Therefore the argument that loss of cytosine methylation involves mechanisms similar to the mouse are not fully supported by what is known about the expression of these genes during mouse specification. It is also possible that human might involve repression of Dnmt3b but not Dnmt3a and this might be different to the mouse.

The experiments evaluating Prdm14 in human germline specification are confusing and not supported by any functional data. Levels of Prdm14 are very high in undifferentiated human pluripotent stem cells, therefore although the expression of Prdm14 is lower in the in vitro PGC system, it is unclear whether Prdm14 is completely lacking as the authors suggest or whether it is simply reduced. The authors should supply functional data backing up the provocative hypothesis that Prdm14 is not required for human germline development in the same way it is required in the mouse.

There are too many places where the authors suggest their data supports previous work in the mouse ESC to PGCLC differentiation model, however only a small number of examples are given. If the authors intend to follow this line or argument a microarray/RNA-Seq experiment comparing PGCLCs differentiated from mouse EpiLCs and PGC-like cells differentiated from human should be performed on the same platform so as to avoid batch effects that could significantly impact the conclusions.

Minor: Small spelling errors are found in places, for example B

Referee #2:

The manuscript by Sugawa et al. explores a condition under which human pluripotent stem cells differentiate into primordial germ cell-like cells in vitro, and examines the process of hPGC-like cell differentiation by a variety of methods. The following points need to be addressed before considering the publication of this work in the EMBO Journal.

1. In Figure 2A, the authors used STELLA 5'UTR-EGFP expression as a reporter of PGC-like cells. Accordingly, STELLA-EGFP expression was detected from day 6 upon differentiation and was increased until day 9. On the other hand, in the later experiments, the authors used c-KIT as a marker of PGC-like cells. With this marker, PGC-like cells were detected at the maximum percentage on day 4 and mostly disappeared until day 8. These data are clearly inconsistent and need to be clarified. First, the authors should analyze the expression of STELLA-EGFP shown in Figure 2A by FACS. Second, the authors should re-analyze the PGC-like cell differentiation process (Figure 2C and Sup. Figure 2A) using other markers such as SSEA1. Third, STELLA-EGFP expression should be evaluated, as shown in Sup Figure 2A, in the time course from day 0 to day 8 or 9 to examine the correlation between the EGFP expression and c-KIT positive population. Without these evaluations, STELLA-EGFP data provides no more than confusion, thus better to be removed.

2. In Figure 1D, the expression of T does not seem to correlate with the BLIMP1 expression. The authors should provide better images and more quantitative data, e.g., percentage of cells co-expressing BLIMP1 and T. Co-staining of T and BLIMP1 should also be done in Figure 4C.

3. In Figure 3B, only a subpopulation of c-KIT+ cells express BLIMP1. The authors should provide more quantitative data, e.g., percentage of BLIMP1 or STELLA positive cells in the cKIT+ population.

4. In Figure 3C, the authors should state which time point of samples were used for the analysis.

5. Figure 3E is not conclusive. It should be removed from the main figures.

6. In Sup Figure 3, after PGC-like cell induction, the percentage of c-KIT positive population is the highest in 20% KSR condition. However, in Figure 4D, BLIMP1 positive cells are the fewest and lack OCT4 expression. The authors should provide explanation. What is the identity of the c-KIT positive cells induced with 20% KSR?

7. In Figure 4D, with 10% KSR, the total of OCT4+ and BLIMP1+ cells exceeds 100%, in the absence of OCT4+ BLIMP1+ cells. Why?

8. Overall, IF images are poor in the quality and resolution, which should be improved.

Referee #3:

Several studies have previously reported induction of PGC-like cells from human ES cells with the detection of germ cell genes, including BLIMP1, DAZL and NANOS3, some of which are also found in mouse PGCs. Here the authors have attempted to follow some of the recent work in mice to re-visit this question. They show in particular absence of SOX2 in human PGCs, which was shown previously. A recent paper in Stem Cell Reports (2014) on PGCs from human ES cells also suggests downregulation of SOX2. This study also indicates reduced levels of PRDM14 in human PGCs.

Introduction

Two key points need to be considered objectively in the Introduction;

1.Page3, 1st para, last sentence. Unfortunately, contrary to what is stated, Prdm14 alone does and can induce PGCLC in vitro as shown by Nakaki et al (2013), and its is also critical for PGC specification in vivo in mice.

2. Page 4, 1st para: Clear statements are required for the role of Prdm14 in pluripotency of both mouse and human ES cells, with its second distinct role in PGC specification in mice. The work of Ma et al., on mouse ES cells should also be cited here. PRDM14 represses differentiation of mouse and human ES cells, and in human ES this includes repression of PGC genes such as NANOS3. This does not imply that PRDM14 in humans might not have a role in PGCs.

Results

1. Page5, 1st para: The authors prepared a human ES cell reporter line with STELLA-GFP with a short transgene. Are the observation based on a single human reporter ES line? The use a short STELLA-GFP transgene in random genomic sites could affect expression due to position effects, and importantly cause ectopic expression. Some evidence should be presented to rule this out. Many prefer a knock-in for reporter lines to rule out these problems.

2. Fig1B. The figure indicates that an increase in Activin A alone induces expression of the genes shown here, without the addition of any BMP4 (red line in the figure)? There is also an increase in the expression of STELLA. Are there any STELA/BLIMP1 positive cells in this population?

3. Fig1C/D: Many different cell types express BLIMP1 and T; what proportions of cells show OCT4/BLIMP1 expression, with or without T? What proportion and which of these cells precisely are considered to be PGC precursors? These heterogeneous cells may not all be 'PGC precursors', and they should be better defined

4. Page 8, Last paragraph. " Differentiation of Precursor Cells". What is meant by precursor cells (see 3 above)?

5. Fig 2 B. Are the GFP positive cells also all positive for STELLA by immunofluorescence to rule out ectopic expression (see comment 1 above)? The study claims 'technical problems', which prevented isolation of GFP positive cells by FACS; this should be explained because the reporter line is important for this work.

6. Page 9. Initially, SSEA1+/cKIT+ cells were used to detect STELLA+ve cells (on Page 5). Are the cells identified by TRA-1-81+/cKIT+ similar to the cells isolated by SSEA1/cKIT?

7. Page 9, First para. The isolation of cells by FACS and their characterisation is a critical point. The cKIT+ ve cells regardless of TRA-1-81 are GFP positive but not those identified by cKIT alone? FACS sorted SSEA1+/cKIT+ cells should be compared with the TRA-1-81/cKIT+ve population, and the GFP positive cells in different populations should be checked by immunofluorescence for STELLA to confirm which cells and what proportion are BLIMP1/STELLA positive, to indicate correspondence between the reporter and protein. Without further characterisation it is not possible to conclude how reliable TRA-1-81 and cKIT are for PGCLs. The images in Fig2B are meant to imply correspondence with Fig2D, but this needs verification.

8. Fig 2D. The maximum numbers of TRA-1-81+/cKit+ cells are on day 4 (>20%), which is not reflected in GFP+ve on day 4 in 2B. When the numbers decline to 2.2% by day 8, there is a strong GFP single in 2B. (The Legend indicates FACS plots until day 10 but the data shown is until day8). The FACS sorted cells should also be analysed by immunofluorescence fro BLIMP1 and STELLA to confirm the identity of cells.

9. Fig 2C/D. Without additional characterisation of FACS sorted cells, the nature of PGC precursors or PGCLC is unclear, and also therefore the implied progressive differentiation from precursor to specification of PGCLC.

10. Fig3A. This data is open to other interpretations. Does this represent upregulation of BLIMP1/STELLA in all these cells, or is this due to a loss of BLIMP1-cells from the population? Since the c-Kit/Tr-1-8-1 cells in Fig 2D are declining at day 6, the data in Fig 3A on these cell populations needs explanation.

11. Fig 3B. Is this a representative image (and on what day?) and what proportions of cells are positive for BLIMP1 and STELLA together, and for the two markers individually, as well as with or without OCT4. The experiments need to provide clear indications of the precise numbers of 'precursors', and finally the PGC-like cells that resulted from the experiment.

12. Fig 3E. Since the results of this experiment are negative, it is important to carry out a genetic experiment, such as knockdown of BLIMP1 and PRDM14 to determine how this affects differentiation of cells.

13. Page 16: PRDM14. The data presented on the downregulation of PRDM14 transcripts does not necessarily reflect the levels of protein present in cells. There seems to be some on going transcription of PRDM14. This could also mean that human PGCs require lower levels of PRDM14. Additional evidence is needed on this.

12. The work on WNT3A (pages 11-14) can be described briefly and with greater clarity.

Discussion

The results of some experiments need to be confirmed as indicated above for the conclusions.

1.Page16, 2nd para. It is not clear if LIF is involved in the expression of pluripotency genes in PGCs in mice in vivo. This is the role of Prdm14 in mice. To state that LIF is important for the expression of pluripotency genes (instead of PRDM14) in PGCLCs in human requires evidence, perhaps with the use of specific inhibitors. Detection of LIFR alone is insufficient. A recent paper in Stem Cell Reports suggests that SOX2 may be repressed by BLIMP1 in PGC like cells to repress neuronal differentiation. The absence of SOX2 may not be linked to the presence or absence of PRDM14.

2. What is the optimum number (in %) of PGCLC obtained from these experiments as verified by BLIMP1/OCT4/STELLA co-expression in isolated cells? And what % of the so-called precursors is converted into PGCLC? The transitions are strongly implied but the characteristics and numbers of cells are unclear, especially as BLIMP1 and T are expressed many other cell types.

1st Revision - authors' response 25 July 2014

Point by point response to reviewers:

We would like to express our gratitude to the three referees for providing valuable suggestions that improve and strengthen our manuscript. We have used these comments as the basis for revising the manuscript and added substantial experimental results to support our findings. Here we address the reviewers' specific comments.

Referee #1:
The authors have provided a new approach for differentiating human germ cells from human pluripotent stem cells. The findings are in most cases solid and the hypothesis that Prdm14 is not associated with human germline development is provocative. The authors need to provide some functional evidence on the role (or not) of Prdm14 in human germline development.

Introduction:
It is hard to conceive how 10-15 weeks corresponds to mid to late stages of development in humans because gestation is 40 weeks, wouldn't it be early?

Response 1. *10-15 weeks corresponds certainly to an early stage of human development. We have corrected this error in the text.*

In the introduction the authors list a number of genes in common between mouse and human, but the reference to all these genes is a review. Can the authors use references from the primary literature for these.

Response 2. *According to the suggestion, we added the references from the primary literature*

The argument that PRDM14 represses NANOS3 and BMP4 is based upon work using mouse pluripotent cells. The phrasing of the argument suggests that the repression of NANOS3 by PRDM14 is a human phenomenon and is misleading.

Response 3. *This argument is based on the finding of a previous study in which RNAi screening was performed on human ESCs (Chia et al. Nature. 2010; 468:316-20). The authors demonstrated that PRDM14 protein binds to 2,755 genes, including NANOS3 and BMP4, and these two genes are upregulated by knock-down of PRDM14 in human ESCs. Thus, the authors suggested a negative regulatory effect of PRDM14 on NANO3 and BMP4 in humans. Please refer to page 319 and supplementary figure 20 in the mentioned report.*

Results:
The Activin and BMP induction protocol is very interesting and the increase in an intermediate cell between mesoderm and germline. However an alternate interpretation is that it could also mean a heterogeneous mix of mesoderm and germ line cells. Can the authors address heterogeneity? Can the authors supply a better triple stain with T in Figure 1D. Also, can the authors provide quantification on the number of triple positive cells at day 2 based on number of cells plated.

Response 4. *We provided higher resolution images (new Fig. 1) and quantified the* OCT4/BLIMP1 *and* OCT4/BLIMP1/T *stained cell populations. As shown in the new Fig.1D, we observed ca. 2% of* OCT+/BLIMP+/T+ *cells and 0.7% of* OCT4+/BLIMP1+/T- *cells on day 1 of induction. On day 2 the number of* OCT4+/BLIMP1+/T+ *cells had declined to ca. 0.5%, while the portion of* OCT4+/BLIMP1+/T- *cells had increased to ca. 5%. The rapid loss of* OCT4+/BLIMP1+/T+ *cells after day 1 und the concomitant increase of* OCT4+/BLIMP1+ *cells by day 2 strongly suggests the transition of* OCT4+/BLIMP1+/T+ *cells into* OCT4+/BLIMP1+/T- *cells and correlates with* the *rapid increase of T expression by day 1, followed by the immediate decline by day 2 (Fig. 1B). These results argue against a heterogeneous mix of mesoderm and germ line cells. We included these data in the manuscript.*

The inability to sort GFP+ cells is concerning. Given the authors use an alternate approach for the remainder of their work, it doesn't make sense to include the STELLA-GFP reporter in Fig 1A or 2B or 4B and C bottom panel. Without sorting results, the specificity of this reporter cannot be

appropriately validated.

Response 5. *We agree with the reviewer that the STELLA-GFP reporter is not appropriately validated. Since our alternative approach using TRA-1-81 and c-KIT proved reliable, we removed all STELLA-GFP reporter data and revised Figures and text accordingly.*

Figure legend for 2D indicates that differentiation is shown up to day 10, however the last day indicated is day 8, is it day 10 or day 8.

Response 6. It *is day 8. We regret this mistake and corrected the figure legend accordingly.*

The 5mC immunofluorescence stain in Figure 3D shows two different types if morphologies with iPS cells being spread out whereas the putative PGCs are bunched together making interpretation difficult. Can the authors show loss of 5mC by an alternative technique or different image where the representative cells between control and experiment are similarly placed relative to each other.

Response 7. *According to the suggestion, we provided images with similar cell distribution and hope it is easier now to appreciate the loss of 5 mC in PGCLCs.*

In the ovary experiment shown in Figure 3E, no teratoma formed when PGCs were used for transplantation, but what happened to the human cells? Did they survive but not proliferate, did the e12.5 somatic cells form an ovarianlike structure or were all grafted cells cleared away? Can the authors provide more experimental details to facilitate interpretation of this experiment.

Response 8. *To identify human cells within the ovarian grafts we stained paraffin sections of the explants for human NUMA. As shown in Figure 3E(d), NUMA positive PGC-like cells had survived but apparently did not proliferate nor differentiate much further. These cells were located close to the injection site and were distributed as single cells throughout this part of the tissue. We could not identify ovarian-like structures that could be allocated to the e12.5 somatic cells, as these cells were also mouse cells. In addition, we find it quite difficult to identify ovarian structures unless there are follicles present. It is possible that the original aggregate formed from 12.5 dpc somatic mouse cells and human PGCLCs survived as a whole, but it could also be possible that the 12.5 dpc somatic cells were cleared away. What we can definitely conclude from this experiment is that the PGCLCs survived and, contrary to the grafted iPSCs, did not form teratomas.*

Figure 4C can the authors show higher power images or inserts.

Response 9. *We rearranged and changed all figures in the revised manuscript and removed or added data, respectively. Since part of the information we meant to illustrate in Fig. 4C in the first submission is now contained in Fig. 1, we were able to increase the physical size of Fig.4C to obtain better resolution.*

The reduction in Dnmt1 levels in PGCs (Figure 5D) is surprising as in murine PGCs specification Dnmt1 RNA levels remain constant. Further, both Dnmt3a and Dnmt3b are reduced in mouse PGCs, whereas it appears only Dnmt3b is reduced in human in vitro PGCs with differentiation. Therefore the argument that loss of cytosine methylation involves mechanisms similar to the mouse are not fully supported by what is known about the expression of these genes during mouse specification. It is also possible that human might involve repression of Dnmt3b but not Dnmt3a and this might be different to the mouse.

Response 10. *We agree with the reviewer and revised the text according to the suggestion.*

The experiments evaluating Prdm14 in human germline specification are confusing and not supported by any functional data. Levels of Prdm14 are very high in undifferentiated human pluripotent stem cells, therefore although the expression of Prdm14 is lower in the in vitro PGC system, it is unclear whether Prdm14 is completely lacking as the authors suggest or whether it is simply reduced. The authors should supply functional data backing up the provocative hypothesis that Prdm14 is not required for human germline development in the same way it is required in the mouse.

Response 11. *This is an important point and we performed shRNA knockdown for BLIMP1 and PRDM14 and assessed their influence on TRA-1-81+/c-KIT+ PGC-like cell formation. As shown in Figure 5F, the knock-down of BLIMP1 significantly reduced the percentage of PGC-like cells, despite the moderate efficiency of the knock-down. On the other hand, the knock-down of PRDM14 did not impair the induction of PGC-like cells compared to the control sample. These data further support the hypothesis that PRDM14 is not required for human germline development in the same way it is required in the mouse.*

There are too many places where the authors suggest their data supports previous work in the mouse ESC to PGCLC differentiation model, however only a small number of examples are given. If the authors intend to follow this line or argument a microarray/RNA-Seq experiment comparing PGCLCs differentiated from mouse EpiLCs and PGC-like cells differentiated from human should be performed on the same platform so as to avoid batch effects that could significantly impact the conclusions.

Response 12. *We followed the suggestion and generated mouse PGCLCs according to Hayashi et al. Cell, 2011 and hybridised our own PGCLCs and EpiLCs cells into Illumina gene expression microarrays, handled by the same technician that performed the former human Illumina gene expression microrrays to avoid batch effects. We then used the new transcriptomics mouse PGCLCs and EpiSCs data to substitute the transcriptomics mouse data (download from the GEO database) that we have used in the former version of the manuscript. With the new data we repeated the whole transcriptomics meta-analysis and obtained very similar results as in our former version (shown in Fig. 5). The message remained unchanged. These results demonstrate the robustness of our methodology. To emphasize our results we have devoted a meta-analysis subsection in the results section of the manuscript and we have written the description of our transcritomics meta-analysis approach in the methods section. Additionally, some new results on the expression evolution (obtained by our Illumina microarrays) of gene markers used in the present study, has been added to the Expanded View information material.*

Minor: Small spelling errors are found in places, for example B

Response 13. *We carefully reviewed the manuscript and corrected the errors.*

Referee #2:
The manuscript by Sugawa et al. explores a condition under which human pluripotent stem cells differentiate into primordial germ cell-like cells in vitro, and examines the process of hPGC-like cell differentiation by a variety of methods. The following points need to be addressed before considering the publication of this work in the EMBO Journal.

1. In Figure 2A, the authors used STELLA 5'UTR-EGFP expression as a reporter of PGC-like cells. Accordingly, STELLA-EGFP expression was detected from day 6 upon differentiation and was increased until day 9. On the other hand, in the later experiments, the authors used c-KIT as a marker of PGC-like cells. With this marker, PGC-like cells were detected at the maximum percentage on day 4 and mostly disappeared until day 8. These data are clearly inconsistent and need to be clarified.
First, the authors should analyze the expression of STELLA-EGFP shown in Figure 2A by FACS. Second, the authors should re-analyze the PGC-like cell differentiation process (Figure 2C and Sup. Figure 2A) using other markers such as SSEA1. Third, STELLA-EGFP expression should be evaluated, as shown in Sup Figure 2A, in the time course from day 0 to day 8 or 9 to examine the correlation between the EGFP expression and c-KIT positive population. Without these evaluations, STELLA-EGFP data provides no more than confusion, thus better to be removed.

Response 1. *We agree with the reviewer in point 1 and 3 that the STELLAGFP reporter is not appropriately validated. Since our alternative approach using TRA-1-81 and c-KIT proved reliable, we removed all STELLA-GFP reporter data and revised Figures and text accordingly. To address point 2, we performed gene expression analysis of a subset of germ cell markers and pluripotency markers within the cKIT+/TRA-1-81+ PGCLC population and compared it to the cKIT+/SSEA1+ cell population. As expected and shown in Figure 2D, the analyzed cell fractions*

exhibited a very similar gene expression pattern, indicative of PGCs. This similarity furthermore validates the TRA-1-81/c-KIT marker combination reliable for the isolation of PGCLCs in our system.

2. In Figure 1D, the expression of T does not seem to correlate with the BLIMP1 expression. The authors should provide better images and more quantitative data, e.g., percentage of cells co-expressing BLIMP1 and T. Costaining of T and BLIMP1 should also be done in Figure 4C

Response 2. *We provided higher resolution images (new Fig. 1) and quantified the* OCT4/BLIMP1 *and* OCT4/BLIMP1/T *stained cell populations. As shown in the new Fig.1D, we observed ca. 2% of OCT+/BLIMP+/T+ cells and 0.7% of* OCT4+/BLIMP1+/T- *cells on day 1 of induction. On day 2 the number of* OCT4+/BLIMP1+/T+ *cells had declined to ca. 0.5%, while the portion of* OCT4+/BLIMP1+/T- *cells had increased to ca. 5% Fig. 1D). The rapid loss of* OCT4+/BLIMP1+/T+ *cells after day 1 und the concomitant increase of* OCT+/BLIMP1+ *cells by day 2 strongly suggests the transition of* OCT4+/BLIMP1+/T+ *cells into* OCT4+/BLIMP1+/T- *cells. We included these data in the manuscript.*

3. In Figure 3B, only a subpopulation of c-KIT+ cells express BLIMP1. The authors should provide more quantitative data, e.g., percentage of BLIMP1 or STELLA positive cells in the cKIT+ population.

Response 3. *Figure 3B shows immunostaining of dissociated single cells from whole EBs, which explains the presence of unstained cells. We now provided higher resolution images and also included merged images. Fig. 3B is intended to show that BLIMP1, OCT4, and STELLA are not only expressed at the mRNA level (Fig.3A), but also at the protein level.*

4. In Figure 3C, the authors should state which time point of samples were used for the analysis.

Response 4. *It is day 6. We apologize for the confusion.*

5. Figure 3E is not conclusive. It should be removed from the main figures.

Response 5. *We agree with the reviewer that the figure was not conclusive and therefore added additional data. To identify human cells within the ovarian grafts we stained paraffin sections of the explants for human NUMA. As shown in Figure 3E(d), Numa positive PGC-like cells had survived but apparently had not proliferated nor differentiated much further. These cells were located close to the injection site and were distributed as single cells throughout this part of the tissue. We conclude from this experiment that the PGCLCs survived in the ovarian environment and, contrary to the grafted iPSCs, did not form teratomas.*

6. In Sup Figure 3, after PGC-like cell induction, the percentage of c-KIT positive population is the highest in 20% KSR condition. However, in Figure 4D, BLIMP1 positive cells are the fewest and lack OCT4 expression. The authors should provide explanation. What is the identity of the c-KIT positive cells induced with 20% KSR?

Response 6. *We thank the reviewer for bringing this lack of information to our attention. We had induced PGC-precursors in the presence of 0-20% KSR to investigate the influence of WNT3A on the precursor induction and subsequently on the PGCLC induction (Supplemental Fig.3). We observed that PGCLCs could be induced only from low KSR conditions. The high KSR conditions induced a cell population that did not express germ cell markers besides cKIT. Our conclusion was that high KSR conditions drive differentiation into a non-germ cell lineage. We can now demonstrate that these cells belong to the hematopoietic cell lineage (Fig. 4B and C).*

7. In Figure 4D, with 10% KSR, the total of OCT4+ and BLIMP1+ cells exceeds 100%, in the absence of OCT4+ BLIMP1+ cells. Why?

Response 7. *The cell counting was performed using Image J and it is sometimes difficult for the software to distinguish individual cells if located in close proximity to each other. Since all the important information we would like to show is contained in the new Fig. 4D, we did not include the graph in the new Figure.*

8. Overall, IF images are poor in the quality and resolution, which should be improved.

Response 8*: We followed the recommendation and improved the quality of the images.*

Referee #3:

Several studies have previously reported induction of PGC-like cells from human ES cells with the detection of germ cell genes, including BLIMP1, DAZL and NANOS3, some of which are also found in mouse PGCs. Here the authors have attempted to follow some of the recent work in mice to re-visit this question. They show in particular absence of SOX2 in human PGCs, which was shown previously. A recent paper in Stem Cell Reports (2014) on PGCs from human ES cells also suggests downregulation of SOX2. This study also indicates reduced levels of PRDM14 in human PGCs.
Introduction

Two key points need to be considered objectively in the Introduction; 1.Page3, 1st para, last sentence. Unfortunately, contrary to what is stated, Prdm14 alone does and can induce PGCLC in vitro as shown by Nakaki et al (2013), and its is also critical for PGC specification in vivo in mice.

Response A. *The study by Nakaki et al. demonstrated that overexpression of Prdm14 alone in EpiLCs induces PGCLCs, while the same approach in ESCs did not do so, but rather stabilized STELLA expression in the ES state. We think this is indicative of a different mechanism of action that depends on the cell state. In fact, Nakaki et al. also mentioned in their paper (page 223, second paragraph, last sentence), "Although BP14A induced EpiLCs robustly into a PGC- like state, BP14A induction in ES cells resulted in a peculiar phenotype: intense SC activation with no BV (Expanded View information Fig. E3 c, d), demonstrating that a proper cellular context (for example, a proper epigenetic background, presence/absence of trans-acting factors) is essential for the robust induction of a PGC-like state by TFs."*

2. Page 4, 1st para: Clear statements are required for the role of Prdm14 in pluripotency of both mouse and human ES cells, with its second distinct role in PGC specification in mice. The work of Ma et al., on mouse ES cells should also be cited here. PRDM14 represses differentiation of mouse and human ES cells, and in human ES this includes repression of PGC genes such as NANOS3. This does not imply that PRDM14 in humans might not have a role in PGCs.

Response B. *We agree with the reviewer, this needs to be considered. Accordingly we revised the text on page 4 and cited the work of Ma et al.*

Results

1. Page5, 1st para: The authors prepared a human ES cell reporter line with STELLA-GFP with a short transgene. Are the observation based on a single human reporter ES line? The use a short STELLA-GFP transgene in random genomic sites could affect expression due to position effects, and importantly cause ectopic expression. Some evidence should be presented to rule this out. Many prefer a knock-in for reporter lines to rule out these problems.

Response 1. *We do have data from independent cell lines, but since the STELLA-GFP reporter is not appropriately validated and our alternative approach using TRA-1-81 and c-KIT proved reliable, we removed all STELLAGFP reporter data and revised Figures and text accordingly.*

2. Fig1B. The figure indicates that an increase in Activin A alone induces expression of the genes shown here, without the addition of any BMP4 (red line in the figure)? There is also an increase in the expression of STELLA. Are there any STELA/BLIMP1 positive cells in this population?

Response 2. *We performed protein staining for OCT4, BLIMP1 and STELLA on day1 and day 2 of the differentiation, but never detected any STELLA+ cells in those cultures. Activin A alone induced gene expression of OCT4, NANOG and STELLA slightly, which might be explained by the stimulatory effect Activin A has on hESCs, as has recently been reported (Duggal et al., SCD Vol. 22, No 23, 2013). This report demonstrated, that Activin A-derived ESC lines possess high*

inherent expression levels of pluripotency markers and early PGC markers (amongst them STELLA and c-KIT), which may prone them towards germ cell differentiation. One would expect that the same it true for iPSC. We referred to this report in the discussion and added the citation.

3. Fig1C/D: Many different cell types express BLIMP1 and T; what proportions of cells show OCT4/BLIMP1 expression, with or without T? What proportion and which of these cells precisely are considered to be PGC precursors? These heterogeneous cells may not all be 'PGC precursors', and they should be better defined

Response 3. *We define PGC precursors as OCT4+/BLIMP1+/T(+/-) cells in our system. As shown in the new Fig.1C and 1C2 the percentage of O+/B+/T+ and O+B+T- cells in this experiment was 0.49% and 5.31%, respectively. The rapid loss of O+/B+/T+ cells after day 1 und the concomitant increase of OCT+/BLIMP1+ cells by day2 (new Fig. 1B) strongly suggests a transition in these cells. The rapid increase of T expression by day 1, followed by the immediate expression decline by day 2 argues against a heterogeneous mix of cells. Accordingly we specified the precursor population in the manuscript.* 4. Page 8, Last paragraph. " Differentiation of Precursor Cells". What is meant by precursor cells (see 3 above)?

Response 4. *As outlined in response 3 above, we define PGC precursors as OCT4+/BLIMP1+/T(+/-) cells.*

5. Fig 2 B. Are the GFP positive cells also all positive for STELLA by immunofluorescence to rule out ectopic expression (see comment 1 above)? The study claims 'technical problems', which prevented isolation of GFP positive cells by FACS; this should be explained because the reporter line is important for this work.

Response 5. *We agree with the reviewer that ectopic expression cannot be ruled out, which was another reason for us to switch to an alternative method. As already mentioned in response 3, we obtained reliable data using TRA-1-81 and cKIT (please also see response 6 below) and therefore omitted the STELLA-GFP reporter data.*

6. Page 9. Initially, SSEA1+/cKIT+ cells were used to detect STELLA+ve cells (on Page 5). Are the cells identified by TRA-1-81+/cKIT+ similar to the cells isolated by SSEA1/cKIT?

Response 6. *To address this point and to ensure the germ cell identity of the isolated cell population, we performed gene expression analysis of a subset of germ cell markers and pluripotency markers within the cKIT+/TRA-1-81+PGCLC population and compared it to the cKIT+/SSEA1+ cell population. As expected and in the new Fig. 2, the analyzed cell fractions exhibited a very similar PGC marker expression pattern, demonstrating the close similarity of the two cell populations and the suitability of this marker combination for the PGCLC isolation. We also point this out in the manuscript.*

7. Page 9, First para. The isolation of cells by FACS and their characterisation is a critical point. The cKIT+ ve cells regardless of TRA-1-81 are GFP positive but not those identified by cKIT alone? FACS sorted SSEA1+/cKIT+ cells should be compared with the TRA-1-81/cKIT+ve population, and the GFP positive cells in different populations should be checked by immunofluorescence for STELLA to confirm which cells and what proportion are BLIMP1/STELLA positive, to indicate correspondence between the reporter and protein. Without further characterisation it is not possible to conclude how reliable TRA-1-81 and cKIT are for PGCLs. The images in Fig2B are meant to imply correspondence with Fig2D, but this needs verification.

Response 7. *We agree with the reviewer. In the context of comment and response 5 and 6 we feel more confident about the TRA-1-81+/cKit+ data than the STELLA-GFP reporter data. Accordingly we did not include the STELLA-GFP data in the revision of this manuscript.*

8. Fig 2D. The maximum numbers of TRA-1-81+/cKit+ cells are on day 4 (>20%), which is not reflected in GFP+ve on day 4 in 2B. When the numbers decline to 2.2% by day 8, there is a strong GFP single in 2B. (The Legend indicates FACS plots until day 10 but the data shown is until day8). The FACS sorted cells should also be analysed by immunofluorescence fro BLIMP1 and STELLA to confirm the identity of cells.

Response 8. *The FACS plots were generated until day 8. We corrected the error in the legend and apologize for the confusion. The GFP signal in EBs at day 8 might be the result of residual GFP protein that had not yet been degraded, as GFP is usually quite stable The intensity of this signal might also reflect ectopic expression as suggested above. However, we have removed all GFP-reporter data.*

9. Fig 2C/D. Without additional characterisation of FACS sorted cells, the nature of PGC precursors or PGCLC is unclear, and also therefore the implied progressive differentiation from precursor to specification of PGCLC.

Response 9. *We further characterized d6 PGCLCs by means of gene expression analysis, methylation analysis, and ovarian transplantation in mice, and the corresponding data are presented in Figure 3. Our revised manuscript also contains additional analysis of the ovarian explants and demonstrates that PGCLCs do not form teratomas but survive in the ovarian environment. In addition, we performed microarray analysis of the d2 precursor population and d4 and d6 PGCLCs (Figure 5), which demonstrated with statistical significance of gene ontology terms the downregulation of genes associated with neural differentiation, the temporal upregulation of somatic mesodermal genes and expression of core PGC genes. These data strongly support the specification of PGCLCs in our system.*

10. Fig3A. This data is open to other interpretations. Does this represent upregulation of BLIMP1/STELLA in all these cells, or is this due to a loss of BLIMP1-cells from the population? Since the c-Kit/Tr-1-8-1 cells in Fig 2D are declining at day 6, the data in Fig 3A on these cell populations needs explanation.

Response 10. *BLIMP1 and STELLA are upregulated in TRA-1-81+/cKIT+ cells. Downregulation of those genes in the cKIT- population might be due to loss of Blimp1+ cells that are not committed to the germ lineage and are not supported by the PGCLC induction medium. The decline of TRA-1-81+/cKIT+ cells by day 6 in Fig. 2D may also be explained by the medium condition, as a similar effect has been reported for mouse PGCLCs (Hayashi et al., Cell, 2011) under this media condition.*

11. Fig 3B. Is this a representative image (and on what day?) and what proportions of cells are positive for BLIMP1 and STELLA together, and for the two markers individually, as well as with or without OCT4. The experiments need to provide clear indications of the precise numbers of 'precursors', and finally the PGC-like cells that resulted from the experiment.

Response 11. *The images depict cells from dissociated EBs from a "high yield" differentiation experiment, i.e. more than 20% TRA-1-81+/cKIT+ PGCLCs at d6. We did not perform a statistical cell count of all the different marker combinations, since the yield of PGCLCs fluctuated between 5 and 25% in different experiments, which we attribute to variations in the quality of the initial ESC cultures. This variation makes the determination of precise cell numbers, which are statistically sound, extremely difficult. We now provided higher resolution images and also included merged images. Fig. 3B is intended to show that BLIMP1, OCT4, and STELLA are not only expressed at the mRNA level (Fig.3A), but also at the protein level. We hope that the substantial experimental data that we added in this revision compensate for the lack of this requested information.*

12. Fig 3E. Since the results of this experiment are negative, it is important to carry out a genetic experiment, such as knockdown of BLIMP1 and PRDM14 to determine how this affects differentiation of cells.

Response 12: *We agree with the reviewer and performed shRNA knockdown for BLIMP1 and PRDM14 and assessed their influence on TRA-1-81+/c-KIT+ PGC-like cell formation. As shown in Figure 5F, the knock-down of BLIMP1 significantly reduced the percentage of PGC-like cells, despite the moderate efficiency of the knock-down. On the other hand, the knock-down of PRDM14 did not impair the induction of PGC-like cells compared to the control sample. These data further support the hypothesis that PRDM14 is not required for human germline development in the same way it is required in the mouse. We integrated this data set in the manuscript (Fig. 5x).*
In the chimeric ovarian transplantion experiments we observed that teratomas had formed from the

reconstituted ovaries generated from iPSCs (n=2) (Fig. 3E(a,b)), but not from the PGCLCs (n=10) (Fig.3E(c)), indicating that those cells are not pluripotent. To identify human cells within the ovarian grafts we stained paraffin sections of the explants for human NUMA. As shown in Figure 3E(d), NUMA positive PGC-like cells had survived in the reconstituted ovaries but apparently had not proliferated nor differentiated much further. These cells were located close to the injection site and were distributed as single cells throughout this part of the tissue. However, we also did not observe mature PGC-like cells, which can be attributed to a likely nonpermissive environmental niche.

13. Page 16: PRDM14. The data presented on the downregulation of PRDM14 transcripts does not necessarily reflect the levels of protein present in cells. There seems to be some on going transcription of PRDM14. This could also mean that human PGCs require lower levels of PRDM14. Additional evidence is needed on this.

Response 13. *We agree, however the knock-down of PRDM14 did not negatively influence the PGCLC formation and this observation argues against the possibility that human PGCs require lower levels of PRDM14.* 12. The work on WNT3A (pages 11-14) can be described briefly and with greater clarity.

Response 14. *We agree with the reviewer and significantly shortened this section to transmit our message more comprehensively*

Discussion

The results of some experiments need to be confirmed as indicated above for the conclusions.
1.Page16, 2nd para. It is not clear if LIF is involved in the expression of pluripotency genes in PGCs in mice in vivo. This is the role of Prdm14 in mice. To state that LIF is important for the expression of pluripotency genes (instead of PRDM14) in PGCLCs in human requires evidence, perhaps with the use of specific inhibitors. Detection of LIFR alone is insufficient.

Response. *We followed this advice and performed PGCLC induction in the presence of JAK inhibitor. The induction efficiency was not affected in the presence of JAK inhibitor, which demonstrated that LIF is indeed not responsible for the expression of pluripotency genes in human PGCLCs. We do not show the data, but removed the statement from the text.*

A recent paper in Stem Cell Reports suggests that SOX2 may be repressed by BLIMP1 in PGC like cells to repress neuronal differentiation. The absence of SOX2 may not be linked to the presence or absence of PRDM14.

Response. *We addressed this point in the discussion and cited the publication.*

2. What is the optimum number (in %) of PGCLC obtained from these experiments as verified by BLIMP1/OCT4/STELLA co-expression in isolated cells?

Response. *The optimum number we obtained was 25%, however, we experienced a strong fluctuation in yield from different experiments, with yields as low as 5% and as high as 25%. We did not verify by* BLIMP1/OCT4/STELLA *co-expression, since we routinely validated the PGCLCs by qPCR. The new Fig.3B shows* OCT4, BLIMP1 *and* STELLA *staining of PGCLCs at higher resolution now and demonstrates co-expression of the three proteins in PGCLCs.*

And what % of the so-called precursors is converted into PGCLC? The transitions are strongly implied but the characteristics and numbers of cells are unclear, especially as BLIMP1 and T are expressed many other cell types.

Response. *The % of precursor cells that converted into PGCLCs could not be determined, as both cell types are proliferating and we could not utilize a specific marker for tracking the precursors. We provided however immunostainings of* OCT4/BLIMP1 *and* OCT4/STELLA *to demonstrate the expression of the three proteins in PGCLCs (Fig.3B). Furthermore, we compared SSEA1+/cKIT+ FACS fractions of PGCLCs with TRA-1-81+/cKIT+ by qPCR and obtained a very similar gene expression pattern of specific PGC-and pluripotency markers, demonstrating the close similarity of*

the two cell populations (Fig.2D). In addition, PGCLCs did not express the hematopoietic markers CD43 and CD45 (Fig. 4C, Control), as did the cKIT+ cells that were generated under high KSR conditions. Together with the micro array analysis we can demonstrate that our PGCLCs possess germ cell characteristics and we can exclude a somatic cell identity.

2nd Editorial Decision (TS-R) 22 August 2014

Thank you very much for your revised paper that was re-assessed by the original referees. With my colleague Alexander Kohlmaier being currently on vacation, I am stepping in as senior editor for the stem cell category of The EMBO Journal as to avoid unnecessary delays.

As you will recognize from the enclosed comments, the decision turns not out to be simple and straightforward. I therefore triggered what we call 'pre-decision cross commenting', an initiative aimed at crystallizing crucial and therefore necessary further demands in case of conflicting recommendations from various referees.
It emerges from the rather consistent comments of refs#1 and #3 that essential controls for knockdown efficiency by various means are indicated to maintain the major premise of the current study. Secondly, the DNA-methylation analyses need to be much further extended and bolstered by comparative and quantitative data as indicated specifically by the comments of ref#3 point 2.
Lastly, with the message that Prdm14 serves a distinct biological function than proposed in the mouse system, this would have to be experimentally supported and clearly presented as such, with convincing revealing PRDM14-depletion that would leave the demethylation capabilities intact.
Overall, one wonders whether complete restructuring of the paper (based upon convincing experimental support) away from a rather method-driven paper and putting this main finding in the center, would better serve this main functional proposal of the study?
In essence, and as you hopefully gather from these lines: the referees as well as the editors recognize the potential value of the work and are willing to pursue the paper further: along and throughout a second round of necessary and major amendments.
Please recognize however, that even the more supportive referees request thorough experimental validation and remain at this stage still unconvinced that this goal has been achieved.

I therefore kindly urge you to determine whether you are willing to invest the demanded experimental time and efforts to eventually develop the study so as to convince refs#1 and #3. Alternatively, it might be a better option to take the study at this point to a less stringent, rather more method-based title.

Please understand that this approach is solely based on the existing uncertainty and aimed at avoiding disappointments/further delays in the process.

Given the current interest and timeliness of the subject, we certainly encourage you to engage in the requested experimental validations and extensions.

Lastly, please do not hesitate to get in touch in case we can be of any further assistance.

REFEREE COMMENTS

Referee #1:

The authors have addressed my major comments and I am very pleased to see that they performed functional studies to examine the role of Prdm14 in germ cell development.

However, the Prdm14 knockdown was not very dramatic, and it is unclear whether the knockdown was evaluated in sorted germ cells or in the starting ESC population before differentiation. If knockdown was evaluated in the ESCs, the authors need to at least show that the knockdown occurred in the TRA-1-81/cKIT+ germ cells that they acquired after differentiation. As it is possible that the cells that developed into germ cells were the ones that escaped knockdown.

An alternate explanation is that Prdm14 may be so critical that low levels are tolerated and a complete null would be necessary to reveal the phenotype.

Referee #2:

Although the authors addressed some of the points raised in the first round of review, there remain many additional points that require clarification, and I do not think that the manuscript is suitable for consideration for publication in The EMBO Journal.

1. In page 7, first paragraph, the authors claimed that the "simultaneous activation of the mesodermal and PGC programs" induced by the combination of BMP4, activin A and bFGF, based on the expression of a mesodermal (T) and a PGC marker (BLIMP1). However, BLIMP1 is not specific to PGCs and also expressed in the endodermal cells. Given the lack of other features characteristic of PGCs (such as STELLA expression or lack of SOX2 expression), this statement is poorly substantiated. Moreover, the authors need to cite the literatures, which support that the combination of the cytokines (Activin, BMP4 and bFGF) can in fact induce the mesodermal lineage and discuss how the authors' method is different from others that induces somatic mesodermal or endodermal lineage.

2. In both the Abstract and the Results, the authors argued that OCT4(+)BLIMP1(+) cells are the mesoderm-committed PGC precursors. The authors need to provide evidence that STELLA/BLIMP1-expressing PGCLCs are in fact derived from OCT4(+)BLIMP1(+) cells but not from other populations present in the day2 PGC precursor culture. In Figure 1C, the dominant population in both day 1 and day 2 of the PGC precursor induction appears to be the OCT4(+)BLIMP1(-)T(+) or the OCT4(+)BLIMP1(-)T(-) cells, which are mixed with the OCT4(+)BLIMP1(+)T(+) and the OCT4(+)BLIMP1(+)T(-) cells. These data may suggest the presence of heterogeneous mixture of germ, mesodermal and perhaps endodermal lineage, contrary to the authors claim. The dynamics of T expression as stated by the authors ("rapid increase of T expression by day1, followed by the immediate decline by day2") does not negate the possibility that the population consists of the heterogeneous mixture of several lineages.

3. In page 8, paragraph 2, the author stated that the gene expression dynamics during the induction of PGC precursors are developmentally conserved between the human and mouse species. This claim is not substantiated by the data provided by the authors.

4. In Figure 1C, a) the T/Hoechst image in the first raw of upper panel does not correspond to the other 3 images, judging from the pattern of nuclear staining. The authors should provide appropriate images, b) No BLIMP1 positive cells could be found in the day 1 image in the upper panel. Why? c) furthermore, the percentage of cells expressing T seems higher in day 2 than in day 1, which appears contradictory to the argument by the authors that the percentage of the cells expressing OCT4, BLIMP1 and T decreased in day 2 than in day 1, d) additionally, the color-combination in the lower panel should be changed because it is impossible to distinguish triple- from double-stained cells in the current version.

5. Further cultivation of day 2 PGC precursors by BMP4 induces a fairly indistinct c-KIT(+)TRA-1-81(+) population, which appears to be largely overlapped with the c-KIT(-)TRA-1-81(intermediate) population. In particular, the upper right quadrant fraction (i.e., c-KIT(+)TRA-1-81(+) fraction) is essentially indistinguishable from lower right quadrant in day 6 of PGCLC induction (These fractions were used for the subsequent gene expression analysis). Accordingly, quadrants in Figure2B and C set by the authors appear somewhat arbitrary. Fluorescence intensity of the overall c-KIT(-) population appears fluctuating during the time course, which also raises the concern regarding the setting of the quadrant lines separating the positive and negative fractions. The authors need to address these points. The authors also need to show the negative control (isotype-matched control antibody staining) for each FACS data to justify the quadrant lines.

6. In Figure 2D, a) the authors should show the FACS plots of SSEA1/cKIT staining during the induction process and clarify the population sorted for the expression analysis, b) although the authors stated in the text "a very similar gene expression pattern", the data appear to indicate significantly lower expression of PGC markers in SSEA1/cKIT-positive cells than in TRA1-81/cKIT-positive cells. Why are they so different? The authors should analyze the induced cells by triple staining with cKIT, SSEA1 and TRA1-81, c) the data of pluripotency markers, such as OCT4, NANOG or SOX2 are not provided despite the statement that "we performed gene expression

analysis for a subset of germ cell markers and pluripotency markers." Does TEX13B represent a germ cell marker? The expression levels should also ideally be presented as dCT value and the gene expression level needs to be compared between iPSCs, cKIT(+)TRA1-81(+) and cKIT(+)SSEA1(+) population.

7. Figure 3B shows that only a subpopulation of c-KIT(+)TRA1-81(+) cells express BLIMP1. It is unclear why immune-staining of dissociated single cells from whole EBs explains the presence of BLIMP1-unstained cells while these cells are clearly positive for OCT4. This needs to be explained and the authors need to provide more quantitative data.

8. Figure 3D is the only data supporting the global DNA demethylation in PGCLCs, therefore needs to be carefully validated. The immunostaining of 5mC on iPSCs and PGCLCs needs to be done either side-by-side in the same slide or with the same positive control in each slide. Also, the anti-5mC antibody is the mouse monoclonal IgG antibody (amm99021; Aviva), thus the secondary antibody used for the immunostaining protocol could react with both anti-5mC primary antibody and anti-c-KIT primary antibody (mouse IgG) used for the sorting, which potentially prevents the specific recognition of 5mC expression.

9. In Figure 3E(iv), the authors need to provide images with higher resolution and/or magnification, since it is unclear whether the pattern of NUMA staining is specific or not. Please also provide the scales in Figure 3E (iii) and (iv).

10. In Figure 4B, the edge of the contour plots extends out of the frame. PMT voltage or gains needs to be adjusted appropriately.

11. In Figure 5C, the expression levels of OCT4 in both PGCLCs and iPSCs are markedly low compared with other pluripotency markers. This raises a serious concern on the pluripotency of the iPSC line used in the study. This needs to be explained.

12. Regarding Figure 5F and G, 1) the FACS plots need to be included in the Figures, 2) the authors' statement in the last sentence of page 17 is rather invalid, since the knock down experiment in Figure 2G only reduces PRDM14 expression by half, which is too inefficient to discuss the different requirement of PRDM14 for germ cell specification between humans and mice, given apparently normal PGC development in PRDM14+/- mice reported in Yamaji et al 2008.

Material and Methods:

In page 23, first paragraph, the sentence "For generation of stable GFP reporter lines...." needs to be removed.

In page 24, second paragraph, the information regarding anti-c-KIT antibody (550412; eBioscience) is likely incorrect.

In page 24, third paragraph, authors need to provide the more detailed information including the methods of attachment of the cells to microslides and conjugated fluorochromes of the secondary antibodies.

In page 29, second paragraph, authors need to provide the more detailed protocol regarding the transfection of shRNA, such as the amount of the shRNA, cell numbers, or the timing when the cells were used for the assay.

Referee #3:

The authors have made a good effort in response to the comments for revision of the paper. Removal of the experiments using Stella-GFP has helped to eliminate some contradictions.

I have few further comments on the revised manuscript for the authors to consider:

1.The authors highlight a potentially important observation on Prdm14 that shows modest expression in human PGC-like cells (not the case in mice), which is emphasized in the title and the

abstract. Since Prdm14 antibodies are commercially available and have previously been used in some studies on human ES cells, it would add significantly to their assertions to have immunofluorescence data for Prdm14 on ESCs, precursors and PGC-like cells. The Prdm14 knockdown study which has no effect on PGC-like cells (a negative result and therefore requires caution), would also be greatly helped by Western blot analysis to show if there is any protein there at all, and to see the effectiveness of the knockdown. This would provide data to support their conclusions.

2. DNA methylation (5mC) and bisulfite sequencing data requires clarification (Fig 3C, D). Figure 2C shows the sequence of PGC-like cell induction. The PGC precursors (day 2 in the figure) upon PGC-induction show a peak response 2 days later (Day4: approx. 20%), and the c-KIT/TRA-1-81 population then dwindles over the next 48h to 2.2 % (day 8 in the Fig). I assume that the cells in Fig 3C, D were from 'day 6' as shown in Fig 2C, that is approximately 96h after the appearance of PGC-like cells. Over this short interval, the cells appear to show an overall decline in 5mC by immunofluorescence (3D), as well as loss of 5mC for some DMRs (3C). This seems like an very abrupt loss of 5mC, especially for the DMRs, which take longer in mice, as this normally only occurs in advanced PGCs when they have entered the gonads and not while they are migrating. What the underlying mechanism might be for the observations in 3C,D requires consideration. It may be of interest to check day2 precursors, and PGC-like cells from an earlier time point for 5mC. This would be of interest in conjunction with the data shown for iPSC. The expectation might be that this process would be more protracted in human compared to mouse, and not as rapid as it appears to be in PGC-like cells in vitro.

3. Page 8: The yield of PGC-like cells from different lines is stated to vary and was as low as 5% depending on the cell line. It would be generally useful to know how many lines were tested and what was the efficiency in each case.

Minor points:

1. Was there any change in the expression of Np5a?

2. The citations could be more balanced

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cross-referee commenting excerpt:

1) The notion that germ cells differentiate from a T positive state was recently proven in the mouse model. Lineage tracing tools were used, which are unavailable in the human system. Unfortunately, the authors de-emphasise the fact that there is a considerable amount of heterogeneity in the early stages of differentiation and only a small proportion of cells appear to be following the hypothesized fate of epiblast like to T-positive state to PGC based on co-expression. I think the authors need to acknowledge this heterogeneity - However, to prove lineage fate - which particularly reviewer 2 seem to prefer to see - is really difficult in the human system, without lineage tracing tools. This could however be acknowledged by the authors while building their case.

2) The methylation analysis in PGCLCs definitely needs to be performed in a quantitative manner, preferably dot blot or Mass Spec or something along these lines.

3) The heart of this paper is the conclusion that PRMD14 has a different function in human germ cell formation. All of the reviewers required more evidence for this - Specifically, proof that knockdown was indeed achieved in the PGCLCs. As pointed out by reviewer 2, FACS plots of control and knockdown cells should also be included in the paper and a more detailed written method for how they performed their knockdown are also very important. The authors should prove lack of function by tying the knockdown (if it really works efficiently and brings the proposed outcome) to the methylation analysis. This is because the hypothesized role for Prdm14 in mouse germ cells is to repress the machinery required for DNA methylation and enable the global loss of 5mC from the germ line genome. If PRDM14 has a different role in human, then a knockdown of PRDM14 would result in germ cells that are capable of demethylation. If the authors can achieve this experiment with quantitation and statistics, the story would be convincing, interesting and suitable for The EMBO Journal.

Additional correspondence (author) 25 August 2014

Thanks a lot for your feedback. The last few days we were struggling a bit how to proceed, especially as Fumihiro Sugawa returned to Japan (we were all confident that we had done what the referees had requested). Our preference would be to go with your suggestion and "take the study at this point to a less stringent, rather more method-based title". Would a title like "Human germ-cell-specification in vitro" be appropriate? What beyond rewriting/restructuring the manuscript would you want us to do?

This way, Yuyong Yoon, who continues this project would have a clean start. Would you then be interested in us submitting to EMBO Journal a functional study on PRDM14 using our system? In such a case we do our best to provide convincing experimental support along and hopefully even beyond the lines set by the referees.

Additional correspondence (editor AK) 02 September 2014

While Dr. Schwarz-Romond is currently out of office for the next two weeks, I have been in contact with him and additionally discussed your request with our chief editor.

We would certainly be very interested to consider a revised and restructured version of your manuscript that transforms the currently slightly more methodologically-oriented manuscript to a more functional study, focuses on PRDM14, uses your new system and takes into consideration the specific requests outlined by Dr. Schwarz-Romond in his letter to you. Publishing such a manuscript would, in my eyes, offer to you as author the largest impact for your work, but requires some involved further-reaching experimentation.

Regarding the second point, we suggested to take the entire study (in its current form) to a less stringent rather more method-based „title“. Such a journal could, for example, be our sister journal EMBO Reports, but such a submission would also require you to address some of the several technical concerns (in this case, more emphasis should be put on addressing the open technical/methodological concerns relating to the in vitro system). We think of this as an alternative to the aforementioned long-term option, but did not mean to say you should change the „paper’s title/heading“.

For this reason, and reading that you are seeking a reasonable and fast solution for publishing the current manuscript (as it is), we would suggest at this point that you send us a point-to-point response to the referee’s comments BUT with a clear view on framing a more methodological version of the current paper that attends to the open technical concerns raised. I would like to add, that this includes responses to referee #2’s comments, which seem to become more important for such a paper. We think that we would need to know the content of such a paper before any commitments could be made.

I hope this response and suggestion are helpful for your decisions how to proceed. Please do contact me with any other questions, I am available for you any time.

Additional correspondence (author) 02 September 2014

Two questions before I meet with the coauthors to discuss how to proceed: we were all a bit surprised that referee 2 came up with quite a list of new experiments. What is the official policy of EMBO J if that happens? Just go ahead with the new list?

Additional correspondence (editor AK) 03 September 2014

The EMBO Journal editors actively police referee comments, and while I asked you for a point-by-point response, we most definitely NOT expect you to experimentally address every single point. At this point we would just like to invite you to respond to each point raised, so that we can return a

more specific suggestion to you on how to move forward. To aid this process, we may also consult with the other referees or outside advisors on specific issues, as necessary. I hope you see that we try to be constructive here, as we value your work highly. And we suggested this procedure in order to be able to evaluate and give you an informed answer whether publishing a more methods-oriented paper might be more appropriate, as laid out in my previous response to you.

I would like to describe also that the EMBO Journal tries to be very hands-on in managing a realistic and constructive editorial process. If you prefer, I can also offer to call you to discuss the measures we have applied towards this goal. Just as an example to demonstrate the efficacy of these policies and the benefits the authors have from this, >95% of manuscripts are now published after 1 round of experimental revision, taking only 77 days on average. Also, >97% manuscripts where we invite a revision are ultimately accepted, which certainly demonstrates our involvement in the interest of the authors.

I thank you for asking these questions indeed, and I hope you see that we wish the editorial process to be transparent and fair. Please do let me know when we can be of any further help at this point, before or after you discuss with your co-authors. I am looking forward to your letter.

Additional correspondence (author) 08 September 2014

We have now tried to answer each point as carefully as possible. We really think that the work deserves to be published in EMBO J. However, as Fumihiro Sugawa in the meantime returned to Japan, and we are not sure if we will ever satisfy referee 2, we would take your advice if you think that we should rather go for EMBO Reports.

Point by point response to reviewers:
We would like to thank the three referees for their time and efforts in evaluating our revised manuscript. Here we address the reviewers' comments.
Referee #1:
The authors have addressed my major comments and I am very pleased to see that they performed functional studies to examine the role of Prdm14 in germ cell development.

However, the Prdm14 knockdown was not very dramatic, and it is unclear whether the knockdown was evaluated in sorted germ cells or in the starting ESC population before differentiation. If knockdown was evaluated in the ESCs, the authors need to at least show that the knockdown occurred in the TRA-1-81/cKIT+ germ cells that they acquired after differentiation. As it is possible that the cells that developed into germ cells were the ones that escaped knockdown.

An alternate explanation is that Prdm14 may be so critical that low levels are tolerated and a complete null would be necessary to reveal the phenotype.

Response: *It has been demonstrated that a decrease of PRDM14 expression in hESCs to 0.25- and 0.33-fold of the transfected cells results in downregulation of pluripotency markers and upregulation of early differentiation marker genes, together with a colony morphology, indicative of the presence of differentiating ESCs (Tsuneyoshi* et al., *BBRC, 2008; Chia* et al., *Nature, 2010). We therefore performed the knockdown on day 2 (d2) of precursor induction to avoid an effect on the pre-differentiation or a bias on the cell lineage. We evaluated the knockdown in d6 PGCLCs. We agree with the reviewer that germ cells could have also developed from cells that escaped the knockdown; however, one would then expect a reduction in the number of PGCLCs, similarly to the reduction observed after BLIMP1 knockdown. To clarify this issue, we can repeat the experiment to improve the knockdown efficiency. We will also include the FACS plots in the Figure. The alternate explanation cannot be ruled out by these* in vitro *experiments, but will be discussed in the manuscript.*

Referee #2:
Although the authors addressed some of the points raised in the first round of review, there remain many additional points that require clarification, and I do not think that the manuscript is suitable for consideration for publication in The EMBO Journal.

1. In page 7, first paragraph, the authors claimed that the "simultaneous activation of the

mesodermal and PGC programs" induced by the combination of BMP4, activin A and bFGF, based on the expression of a mesodermal (T) and a PGC marker (BLIMP1). However, BLIMP1 is not specific to PGCs and also expressed in the endodermal cells. Given the lack of other features characteristic of PGCs (such as STELLA expression or lack of SOX2 expression), this statement is poorly substantiated. Moreover, the authors need to cite the literatures, which support that the combination of the cytokines (Activin, BMP4 and bFGF) can in fact induce the mesodermal lineage and discuss how the authors' method is different from others that induces somatic mesodermal or endodermal lineage.

Response 1: *We based our claim on not only "the expression of a mesodermal (T) and a PGC marker (BLIMP1)", but also on Figs. E2 and E4. However, we agree with the reviewer that the aforementioned claim is insufficiently supported by the text on page 7. We will therefore rephrase the sentence. We will also add citations that support that the combination of Activin A, BMP4, and bFGF induces the mesoderm lineage (Yang* et al., *Nature 2008; Willems* et al., *Differentiation 2008).*

2. In both the Abstract and the Results, the authors argued that OCT4(+)BLIMP1(+) cells are the mesoderm-committed PGC precursors. The authors need to provide evidence that STELLA/BLIMP1-expressing PGCLCs are in fact derived from OCT4(+)BLIMP1(+) cells but not from other populations present in the day2 PGC precursor culture. In Figure 1C, the dominant population in both day 1 and day 2 of the PGC precursor induction appears to be the OCT4(+)BLIMP1(-)T(+) or the OCT4(+)BLIMP1(-)T(-) cells, which are mixed with the OCT4(+)BLIMP1(+)T(+) and the OCT4(+)BLIMP1(+)T(-) cells. These data may suggest the presence of heterogeneous mixture of germ, mesodermal and perhaps endodermal lineage, contrary to the authors claim. The dynamics of T expression as stated by the authors ("rapid increase of T expression by day1, followed by the immediate decline by day2") does not negate the possibility that the population consists of the heterogeneous mixture of several lineages.

Response 2: *As it was already pointed out in our response to the first version of our manuscript, it is extremely difficult to prove lineage fate in the human system without lineage tracing tools. In addition, Fig. 4D also indicates that OCT4+/BLIMP1+ cells are essential for PGCLC formation. However, we acknowledge the reviewer's concern about the presence of heterogeneity in the early stages of differentiation and will revise this point in the Abstract and Results accordingly.*

3. In page 8, paragraph 2, the author stated that the gene expression dynamics during the induction of PGC precursors are developmentally conserved between the human and mouse species. This claim is not substantiated by the data provided by the authors.

Response 3: *We wanted to say that the upregulation of T and BLIMP1 was seen in the mouse and as well in our human system. We will rephrase the sentence.*

4. In Figure 1C, a) the T/Hoechst image in the first raw of upper panel does not correspond to the other 3 images, judging from the pattern of nuclear staining. The authors should provide appropriate images, b) No BLIMP1 positive cells could be found in the day 1 image in the upper panel. Why? c) furthermore, the percentage of cells expressing T seems higher in day 2 than in day 1, which appears contradictory to the argument by the authors that the percentage of the cells expressing OCT4, BLIMP1 and T decreased in day 2 than in day 1, d) additionally, the color-combination in the lower panel should be changed because it is impossible to distinguish triple- from double-stained cells in the current version.

Response 4: a) *We apologize for not being aware of this and will include the appropriate image. b) Again, we need to apologize for including the Hoechst stain instead of the merged image. We will correct this mistake. c) d2 cultures indeed contain OCT4+/T+ cells; however, this cell population does not co-express BLIMP1 and therefore our results do not contradict the observed decline of OCT4+/BLIMP1+/T+ cell numbers on day 2. Yang* et al., *Nature 2008 also reported the presence of an OCT4+/T+ cell population during cardiovascular progenitor differentiation from hESCs that depicted primitive-streak–like cells undergoing commitment to the endoderm lineage. d) We will change the color combination and try to make the differences more obvious; however, in triple stainings, the individual cell populations are usually more difficult to distinguish.*

5. Further cultivation of day 2 PGC precursors by BMP4 induces a fairly indistinct c-KIT(+)TRA-1-81(+) population, which appears to be largely overlapped with the c-KIT(-)TRA-1-81(intermediate) population. In particular, the upper right quadrant fraction (i.e., c-KIT(+)TRA-1-81(+) fraction) is essentially indistinguishable from lower right quadrant in day 6 of PGCLC induction (These fractions were used for the subsequent gene expression analysis). Accordingly, quadrants in Figure2B and C set by the authors appear somewhat arbitrary. Fluorescence intensity of the overall c-KIT(-) population appears fluctuating during the time course, which also raises the concern regarding the setting of the quadrant lines separating the positive and negative fractions. The authors need to address these points. The authors also need to show the negative control (isotype-matched control antibody staining) for each FACS data to justify the quadrant lines.

Response 5: *To confirm appropriate cKIT and TRA-1-81 stainings, we performed FACS first with unstained and isotope controls for positive and negative control cells. For the gating, we used the unstained controls. The setting of the quadrant lines could have also been chosen to be slightly different; nevertheless, within time-course analysis spanning up to 8 days and analysis of different culture conditions, one can expect a certain fluctuation. We chose the presented settings to yield comparable fractions from different experiments.*

6. In Figure 2D, a) the authors should show the FACS plots of SSEA1/cKIT staining during the induction process and clarify the population sorted for the expression analysis, b) although the authors stated in the text "a very similar gene expression pattern", the data appear to indicate significantly lower expression of PGC markers in SSEA1/cKIT-positive cells than in TRA1-81/cKIT-positive cells. Why are they so different? The authors should analyze the induced cells by triple staining with cKIT, SSEA1 and TRA1-81, c) the data of pluripotency markers, such as OCT4, NANOG or SOX2 are not provided despite the statement that "we performed gene expression analysis for a subset of germ cell markers and pluripotency markers." Does TEX13B represent a germ cell marker? The expression levels should also ideally be presented as dCT value and the gene expression level needs to be compared between iPSCs, cKIT(+)TRA1-81(+) and cKIT(+)SSEA1(+) population.

Response 6: *a) We can include the FACS plots of SSEA1/cKIT staining during the induction process in the figure. b) We did not state that the fractions are identical; we tried to validate the cKIT/TRA-1-81 FACS strategy for PGCLC isolation, tested for specific germ cell markers, and observed similar expression data, i.e. expression of particular PGC-specific markers. c) We apologize for this error, and will correct the sentence. d) TEX13B has been reported to be a specific germ cell marker (Sabour* et al.*, HMG 2010). We can also present the expression values as dCT values, but we feel that this would not be ideal. In addition, gene expression values were normalized to iPSCs; however, if this reviewer prefers, we can also change the figure and display iPSC in addition.*

7. Figure 3B shows that only a subpopulation of c-KIT(+)TRA1-81(+) cells express BLIMP1. It is unclear why immune-staining of dissociated single cells from whole EBs explains the presence of BLIMP1-unstained cells while these cells are clearly positive for OCT4. This needs to be explained and the authors need to provide more quantitative data.

Response 7: *The FACS analysis of day 6 PGCLCs demonstrates a certain amount of TRA-1-81+ cells while the number of double positive cells decreases, which could explain the presence of OCT4+/BLIMP1- cells.*

8. Figure 3D is the only data supporting the global DNA demethylation in PGCLCs, therefore needs to be carefully validated. The immunostaining of 5mC on iPSCs and PGCLCs needs to be done either side-by-side in the same slide or with the same positive control in each slide. Also, the anti-5mC antibody is the mouse monoclonal IgG antibody (amm99021; Aviva), thus the secondary antibody used for the immunostaining protocol could react with both anti-5mC primary antibody and anti-c-KIT primary antibody (mouse IgG) used for the sorting, which potentially prevents the specific recognition of 5mC expression.

Response 8: *Technically, the reviewer is right with the dual reactivity of the secondary antibody and the subsequent prevention of specific recognition of 5mC. However, we observed a clear reduction in 5mC staining, which argues against this. If the secondary antibody had reacted with the anti-c-KIT primary antibody, we would have observed an overall staining of all the cells, which was*

not the case, suggesting that the cKIT antibody did not resist the treatment for the 5mC staining. If necessary, we can repeat the staining with another antibody.

9. In Figure 3E(iv), the authors need to provide images with higher resolution and/or magnification, since it is unclear whether the pattern of NUMA staining is specific or not. Please also provide the scales in Figure 3E (iii) and (iv).

Response 9: *We feel that the NUMA staining is specific, as the majority of cells in direct vicinity to the NUMA+ cells are clearly NUMA-. We purposely chose this magnification to demonstrate the dispersed occurrence of NUMA+ cells. However, larger-magnification images can also be provided. We will also add the scale bars.*

10. In Figure 4B, the edge of the contour plots extends out of the frame. PMT voltage or gains needs to be adjusted appropriately.

Response 10: *We kept the PMT voltage on the Tra1-81 channel and all other channels constant, although a small proportion of cells (less than 3% in all instances) appeared on the right edge of the plot. We wanted to make sure that all samples were measured with the same amplification settings, thereby ensuring the comparability of the different series of experiments. Importantly, the appearance of cells on the edges of the plot does not change the statistics.*

11. In Figure 5C, the expression levels of OCT4 in both PGCLCs and iPSCs are markedly low compared with other pluripotency markers. This raises a serious concern on the pluripotency of the iPSC line used in the study. This needs to be explained.

Response 11: *The chip has several probes for one gene, and the dot indicates the probe that exhibits mechanically low binding. It is evident from the heat map, however, that OCT4 is highly expressed. In addition, there is no doubt about the pluripotent state of the iPSC line used in this study, as the same line generated teratomas in the ovarian transplantation experiment shown in Figure 3E.*

12. Regarding Figure 5F and G, 1) the FACS plots need to be included in the Figures, 2) the authors' statement in the last sentence of page 17 is rather invalid, since the knock down experiment in Figure 2G only reduces PRDM14 expression by half, which is too inefficient to discuss the different requirement of PRDM14 for germ cell specification between humans and mice, given apparently normal PGC development in PRDM14+/- mice reported in Yamaji et al 2008.

Response 12: *1) We will include the FACS plots in the Figure. 2) It has been demonstrated that a decrease of PRDM14 expression in hESCs to 0.25- and 0.33-fold of the transfected cells results in downregulation of pluripotency markers and upregulation of early differentiation marker genes, together with a colony morphology, indicative of the presence of differentiating ESCs (Tsuneyoshi* et al., *BBRC, 2008; Chia* et al., *Nature, 2010). We therefore performed the knockdown on day 2 (d2) of precursor induction to avoid an effect on the pre-differentiation or a bias on the cell lineage. We evaluated the knockdown in d6 PGCLCs. We agree with the reviewer that germ cells could have also developed from cells that escaped the knockdown; however, one would then expect a reduction in the number of PGCLCs, similarly to the reduction observed after BLIMP1 knockdown. To clarify this issue, we will repeat the experiment to improve the knockdown efficiency.*

Material and Methods:

In page 23, first paragraph, the sentence "For generation of stable GFP reporter lines...." needs to be removed.

Response: *We will delete this text.*

In page 24, second paragraph, the information regarding anti-c-KIT antibody (550412; eBioscience) is likely incorrect.

Response: *This is correct. The antibody we used was 17-1179.*

In page 24, third paragraph, authors need to provide the more detailed information including the methods of attachment of the cells to microslides and conjugated fluorochromes of the secondary antibodies.

Response: *We will add the requested information in the Methods.*

In page 29, second paragraph, authors need to provide the more detailed protocol regarding the transfection of shRNA, such as the amount of the shRNA, cell numbers, or the timing when the cells were used for the assay.

Response: *For infection of cells, d2 precursor cultures were pretreated with 10 μM Y-27632 dihydrochloride for 1 hr, and thereafter dissociated into single cells by TrypLE. 1 x10^5 cells in 1 ml of precursor induction medium were infected with 60 μl of concentrated virus in 15-ml tubes and incubated at 37°C and 5% CO_2 for 5 hr with occasional mixing. Thereafter, the cells were washed with PBS three times and used for further PGCLC induction. d2 PGCLC induction cultures were used for the assay.*

Referee #3:
The authors have made a good effort in response to the comments for revision of the paper. Removal of the experiments using Stella-GFP has helped to eliminate some contradictions.

I have few further comments on the revised manuscript for the authors to consider:

1.The authors highlight a potentially important observation on Prdm14 that shows modest expression in human PGC-like cells (not the case in mice), which is emphasized in the title and the abstract. Since Prdm14 antibodies are commercially available and have previously been used in some studies on human ES cells, it would add significantly to their assertions to have immunofluorescence data for Prdm14 on ESCs, precursors and PGC-like cells. The Prdm14 knockdown study which has no effect on PGC-like cells (a negative result and therefore requires caution), would also be greatly helped by Western blot analysis to show if there is any protein there at all, and to see the effectiveness of the knockdown. This would provide data to support their conclusions.

Response 1: *We can perform immunofluorescence data for PRDM14 on ESCs, precursors, and PGCLCs. It has been demonstrated that a decrease of PRDM14 expression in hESCs to 0.25- and 0.33-fold of the transfected cells results in downregulation of pluripotency markers and upregulation of early differentiation marker genes, together with a colony morphology, indicative of the presence of differentiating ESCs (Tsuneyoshi* et al., *BBRC, 2008; Chia* et al., *Nature, 2010). We therefore performed the knockdown on day 2 (d2) of precursor induction to avoid an effect on the pre-differentiation or a bias on the cell lineage. We evaluated the knockdown in d6 PGCLCs. We cannot exclude that germ cells could have also developed from cells that escaped the knockdown; however, one would then expect a reduction in the number of PGCLCs, similarly to the reduction observed after BLIMP1 knockdown. To clarify this issue, we can repeat the experiment to improve the knockdown efficiency. We could also perform Western blot analysis to demonstrate the reduction in protein levels.*

2. DNA methylation (5mC) and bisulfite sequencing data requires clarification (Fig 3C, D). Figure 2C shows the sequence of PGC-like cell induction. The PGC precursors (day 2 in the figure) upon PGC-induction show a peak response 2 days later (Day4: approx. 20%), and the c-KIT/TRA-1-81 population then dwindles over the next 48h to 2.2 % (day 8 in the Fig). I assume that the cells in Fig 3C, D were from 'day 6' as shown in Fig 2C, that is approximately 96h after the appearance of PGC-like cells. Over this short interval, the cells appear to show an overall decline in 5mC by immunofluorescence (3D), as well as loss of 5mC for some DMRs (3C). This seems like an very abrupt loss of 5mC, especially for the DMRs, which take longer in mice, as this normally only occurs in advanced PGCs when they have entered the gonads and not while they are migrating. What the underlying mechanism might be for the observations in 3C,D requires consideration. It may be of interest to check day2
precursors, and PGC-like cells from an earlier time point for 5mC. This would be of interest in

conjunction with the data shown for iPSC. The expectation might be that this process would be more protracted in human compared to mouse, and not as rapid as it appears to be in PGC-like cells in vitro.

Response 2: *We could perform 5mC staining of d2 precursor cultures and earlier time points of PGCLC induction.*

3. Page 8: The yield of PGC-like cells from different lines is stated to vary and was as low as 5% depending on the cell line. It would be generally useful to know how many lines were tested and what was the efficiency in each case.
Response 2: *We can add data from 2 additional lines we tested.*

Minor points:

1. Was there any change in the expression of Np5a?
Response: *We can provide this information upon the decision to pursue the 2nd round of revision.*

2. The citations could be more balanced
Response: *We will adjust the citations according to possible changes in the 2nd round of revision.*

…….
cross-referee commenting excerpt:

1) The notion that germ cells differentiate from a T positive state was recently proven in the mouse model. Lineage tracing tools were used, which are unavailable in the human system. Unfortunately, the authors de-emphasise the fact that there is a considerable amount of heterogeneity in the early stages of differentiation and only a small proportion of cells appear to be following the hypothesized fate of epiblast like to T-positive state to PGC based on co-expression. I think the authors need to acknowledge this heterogeneity - However, to prove lineage fate - which particularly reviewer 2 seem to prefer to see - is really difficult in the human system, without lineage tracing tools. This could however be acknowledged by the authors while building their case.

Response 1: *We will acknowledge the presence of heterogeneity in the early stages of differentiation.*

2) The methylation analysis in PGCLCs definitely needs to be performed in a quantitative manner, preferably dot blot or Mass Spec or something along these lines.

Response 2: *We could perform quantification of 5mC and 5hC using mass spectrometry on the samples used in the current manuscript.*

3) The heart of this paper is the conclusion that PRMD14 has a different function in human germ cell formation. All of the reviewers required more evidence for this - Specifically, proof that knockdown was indeed achieved in the PGCLCs. As pointed out by reviewer 2, FACS plots of control and knockdown cells should also be included in the paper and a more detailed written method for how they performed their knockdown are also very important. The authors should prove lack of function by tying the knockdown (if it really works efficiently and brings the proposed outcome) to the methylation analysis. This is because the hypothesized role for Prdm14 in mouse germ cells is to repress the machinery required for DNA methylation and enable the global loss of 5mC from the germ line genome. If PRDM14 has a different role in human, then a knockdown of PRDM14 would result in germ cells that are capable of demethylation. If the authors can achieve this experiment with quantitation and
statistics, the story would be convincing, interesting and suitable for The EMBO Journal.

Response 3: *As pointed out to reviewer 2 and 3, it has been demonstrated that a decrease of PRDM14 expression in hESCs to 0.25- and 0.33-fold of the transfected cells results in downregulation of pluripotency markers and upregulation of early differentiation marker genes, together with a colony morphology, indicative of the presence of differentiating ESCs (Tsuneyoshi* et al., *BBRC, 2008; Chia* et al., *Nature, 2010). As we used non-inducible knockdown constructs, we performed the knockdown on day 2 (d2) of precursor induction to avoid an effect on the pre-*

differentiation or a bias on the cell lineage and evaluated the knockdown in d6 PGCLC cultures. We agree with the reviewers that germ cells could have also developed from cells that escaped the knockdown; however, one would then expect a reduction in the number of PGCLCs, similarly to the reduction observed after BLIMP1 knockdown. To clarify this issue, we could repeat the experiment to improve the knockdown efficiency and include FACS plots of control and knockdown cells, together with a more detailed written method. However, generation of inducible knockdown constructs and repetition of the experiments to demonstrate that knockdown of PRDM14 would result in germ cells that are capable of demethylation, together with quantification and statistics, is quite ambitious, even more considering the time frame for resubmission.

Additional correspondence (editor AK) 12 September 2014

Thank you for your e-mail and for sending us your point-to-point response to the referees' concerns. I have extensively re-discussed your case with the other members of our editorial team.

We appreciate your detailed response to the concerns raised by referees #1 and #3, which constitute the basis for our decision whether the paper can be considered for publication in the EMBO Journal as outlined earlier in Dr. Schwarz-Romond's decision letter and in our recent conversation. We thank you for commenting so clearly also on referee #2's concerns, which helped us to come to a constructive suggestion for the further handling of your manuscript:

Based on the fact that referee #1's and #3's central request to decisively demonstrate genomic demethylation efficiency in more robust PRDM14 loss-of-function conditions will not be met in a revised manuscript, we cannot consider the manuscript for publication in the EMBO Journal, and our strong recommendation to you is at this point indeed to submit a revised version of you manuscript to EMBO Reports.

I have taken the liberty to discuss you case with Dr. Barbara Pauly, senior editor of our sister journal EMBO Reports. I am glad to be able to inform you that EMBO Reports has expressed interest in your study. If you are, thus, interested in this option, with the added advantage of an expedited review/revision process at EMBO Reports that is based on the already existing referees' reports, please get in contact with EMBO Reports/Dr. Pauly directly. Based on the referees' reports and consistent with Dr. Schwarz-Romond's suggestions in his decision letter, Dr. Pauly has let me know already that EMBO Reports is interested to receive a more methods-oriented revised manuscript, that is, without the problematic focus on PRDM14 function. Dr. Pauly further specified that she will send out this more methods-oriented manuscript to the original referees #1 and #3. In my opinion, additionally introducing at least some textual amendments that critically discuss some of the more relevant technical points raised by referee #2 (as you did in the point-to-point response that we received from you) may contribute to the clarity of a revised manuscript and be helpful for the less specialized readership. But please do contact Dr. Pauly directly for all details.

I honestly hope for the rapid publication of your study and that you see positively our editorial handling.

Additional correspondence (author) 28 January 2015

Thank you for your quick response to our letter concerning the aforementioned manuscript.

With regard to the state of revision for the manuscript at this time, we would like to outline the following:

We have generated most of the data requested by all the reviewers that address the technical aspects of the experiments. We are currently performing the last ICC experiments to fulfill all the requirements.

Based on our microarray data, we have now analyzed additional genes regulating DNA methylation, and we have not observed any significant differences—neither in the human, nor in the mouse system. We have also analyzed the expression of Sox17 and other SOX family members (according to Irie et al., Cell) in our precursor- and PGC-like cell populations. We can

clearly demonstrate that SOX17 is highly expressed in both populations, validating the germ cell identity of the generated cells.

The requested quantitative methylation analyses require a large number of cells. We are currently collecting the cell populations from different time points to obtain sufficient material for analysis to include these data in the revision.

As previously discussed additional functional PRDM14 data, like the knockdown experiments, were not pursued for the resubmission.

I hope you will find this outline helpful. I look forward to receiving your assessment.

Additional correspondence (editor BP) 30 January 2015

Thank you for the follow-up letter. I have looked through the information in detail and discussed the dataset again in great detail with colleagues. As you discussed with my colleagues previously, the decisions were largely based on the assessment by referees 1 and 3. While referee 2 is a very experienced and appropriate expert in this area of research, s/he did raise significant additional issues in the last round, which we are (and were) prepared to largely forego.
Referee 3 and also referee 1 in referee cross-commenting agreed on the experimental issues that were requested explicitly for revision.
It appears that many of these issue can be addressed in revision and that the data for these this is already available and ready for resubmission.

In light of this and the significant potential interest of the dataset, we do invite a resubmission to the EMBO Journal.

Please note that we had unfortunately had missed the Cell paper until now. It is unfortunate that we only hear about this paper more than a month after publication, as obviously the priority of significant discoveries erode rapidly as a function of time for a second publication.

Given the situation, we can only proceed, if we can act very rapidly now on both sides:
1) Within ten days, we would require
a) a detailed point-by-point rebuttal including specific reference to all the new data that has been generated in revision and that can be added at this time, including DNA methylation regulator expression and SOX family member expression. You refer to ongoing ICC and quantitative methylation (BIS sequencing) experiments: please provide an estimate if these data can be completed in this time frame; alternatively we want to discuss in how far this data has to be completed for a definitive paper at this stage in the interest of time. We do not require further reaching PRDM14 data. This data should be retained though and the text of the manuscript adjusted accordingly to avoid overstatements.
b) a revised manuscript that includes this data in a form that allows us and expert advisors to rapidly track and adjudicate the new data. Please ensure that the figures and text are revised to final publishable form and address the key points under discussion. Please ensure that the Irie *et al.* (2015) paper is formally cited and discussed.
c) the author checklist (attached) fully completed
d) a short synopsis text with 3-5 bullet points.
e) relevant licence forms, which will be sent by my colleague by separate mail.

2) We will then endeavour evaluate the new data (which may necessitate expert advice specifically restricted to the new data) within 48hours.

3) In the still hypothetical case that we can publish the dataset in the EMBO Journal, we would enter a fast track production process, which can take as little as 10 days.

Please update us when we can expect to receive the above items, so that we can ensure that the process runs as efficiently as possible henceforth.

2nd Revision - authors' response 09 February 2015

Here we address the reviewers' comments to Manuscript EMBOJ-2014-88049R1 and outline the changes in Manuscript EMBOJ-2014-88049R2.

The following common concerns and the concerns raised in the cross-referee commenting excerpt have been removed from the point-by-point response to the individual referees below and are addressed first. In addition, we outline the additional data sets we included in the submitted version, which are not mentioned in the point-by-point response to the reviewers.

Common concerns of referees:
1. Cell heterogeneity: We acknowledged the cell heterogeneity in the early stages of differentiation and revised the abstract, results, and discussion sections accordingly.
2. Knockdown experiment: It has been demonstrated that a decrease of *PRDM14* expression in hESCs to 0.25- and 0.33-fold of the transfected cells results in downregulation of pluripotency markers and upregulation of early differentiation marker genes, together with a colony morphology, indicative of the presence of differentiating ESCs (Tsuneyoshi *et al*, BBRC, 2008; Chia *et al*, Nature, 2010). We therefore performed the knockdown on day 2 (d2) of precursor induction to avoid an effect on the pre-differentiation or a bias on the cell lineage, and evaluated the knockdown in d4 PGCLCs. We agree with the reviewer that germ cells could have also developed from cells that escaped the knockdown; however, one would then expect a reduction in the number of PGCLCs, similarly to the reduction observed after *BLIMP1* knockdown. We also included the FACS plots (Fig. E10). It cannot be ruled out that PRDM14 is so critical that low levels are tolerated and a complete null would be necessary to reveal the phenotype. We addressed these points in the manuscript. As d2 precursor cultures already exhibited reduced *PRDM14* levels, one would have expected a detectable negative effect on PGCLC formation even with moderate knockdown efficiencies.

Cross-referee commenting excerpt:

1. We acknowledged the cell heterogeneity in the early stages of differentiation and revised the abstract, results, and discussion sections accordingly.
2. We did not fulfill the request for a quantitative dot blot or Mass Spec methylation analysis in a dynamic manner; however, we performed 5mC staining on FACS-sorted Tra-1-81$^-$/cKIT$^-$, Tra-1-81$^+$/cKIT$^+$, and Tra-1-81$^+$/cKIT$^-$ cell fractions of d6 PGCLCs and obtained a significant reduction in signal. These data are now presented in Fig. 3D. All fractions were isolated from the same culture and stained side by side to ensure identical treatment of samples. BIS analysis also demonstrated loss of 5mC for some DMRs (Fig. 3C). Taken together, these data strongly suggest ongoing global and locus-specific epigenetic reprogramming in PGCLCs.

3. A comparison of our PGCLCs with 16-week PGCs is helpful to determine whether PGCLCs have indeed acquired a PGC fate. The optimal comparison would have been with early human PGCs, but as in Germany, for legal reasons, we do not have access to such cells, we took cells of the closest stage for which information was available in the databases. We agree with the reviewer that this could be a drawback in such a comparison, as she/he pointed out that the global DNA demethylation might be completed at 16 weeks and this could have an impact on the transcriptome. However, we hope that we could show that it has been useful to do this comparison, as the expression profile of our PGCLCs indeed showed significant similarities with later human PGCs. Furthermore, our comparison also includes newly specified mouse PGC-like cells that are by now quite well characterized and accepted.

Additional data included in this revised manuscript that are not mentioned in the point-by-point response

1. We performed transcriptomics analyses of SOX family members after the *Cell* paper by Irie *et al* was published, and included these data in the manuscript (Fig. E4). SOX17 was highly upregulated in d2 precursors, and this expression was maintained in d4 and d6 PGCLCs, which confirms the PGCLC specification from mesodermal precursors in our system. We cited Irie *et al* appropriately.

2. We included a transcriptomics analysis–based comparison between mouse and human PGCLCs of specific genes that are known to be involved in PGC specification (Fig. 5E). The data demonstrated that *Prdm14* and *Sox2* were downregulated upon ESC differentiation into EpiLCs and thereafter were re-expressed in PGCLCs. In the human system, *SOX2* levels were slightly lower in d2 precursors, significantly decreased in d4 PGCLCs, and were nominal in d6 PGCLCs. On the contrary, *PRDM14* showed very low levels already in d2 precursors and *PRDM14* expression was not upregulated in PGCLCs again. This drop of *PRDM14* was also confirmed at the protein level in precursor cultures (Fig. E11).

Point-by-point response to reviewers:
Referee #1:

However, the Prdm14 knockdown was not very dramatic, and it is unclear whether the knockdown was evaluated in sorted germ cells or in the starting ESC population before differentiation. If knockdown was evaluated in the ESCs, the authors need to at least show that the knockdown occurred in the TRA-1-81/cKIT+ germ cells that they acquired after differentiation. As it is possible that the cells that developed into germ cells were the ones that escaped knockdown.

An alternate explanation is that Prdm14 may be so critical that low levels are tolerated and a complete null would be necessary to reveal the phenotype.

Response: *It has been demonstrated by others that a decrease of PRDM14 expression in hESCs to 0.25- and 0.33-fold of the transfected cells results in downregulation of pluripotency markers and upregulation of early differentiation marker genes, together with a colony morphology, indicative of the presence of differentiating ESCs (Tsuneyoshi* et al, *BBRC, 2008; Chia* et al, *Nature, 2010). We therefore performed the knockdown on day 2 (d2) of precursor induction to avoid an effect on the pre-differentiation or a bias on the cell lineage, and evaluated the knockdown in d4 PGCLCs. We agree with the reviewer that germ cells could have also developed from cells that escaped the knockdown; however, one would then expect a reduction in the number of PGCLCs, similarly to the reduction observed after BLIMP1 knockdown. We also included the FACS plots (Fig. E10). The alternate explanation cannot be ruled out by this* in vitro *experiment and we addressed this point in the manuscript. As d2 precursor cultures already exhibit reduced PRDM14 levels, one would expect a detectable negative effect on PGCLC formation even with moderate K/D efficiencies.*

Referee #2:

1. In page 7, first paragraph, the authors claimed that the "simultaneous activation of the mesodermal and PGC programs" induced by the combination of BMP4, activin A and bFGF, based on the expression of a mesodermal (T) and a PGC marker (BLIMP1). However, BLIMP1 is not specific to PGCs and also expressed in the endodermal cells. Given the lack of other features characteristic of PGCs (such as STELLA expression or lack of SOX2 expression), this statement is poorly substantiated. Moreover, the authors need to cite the literatures, which support that the combination of the cytokines (Activin, BMP4 and bFGF) can in fact induce the mesodermal lineage and discuss how the authors' method is different from others that induces somatic mesodermal or endodermal lineage.
Response 1: *We agree with the reviewer that the aforementioned claim was insufficiently supported by the text on page 7. We therefore included additional information in the results section (first paragraph) and added the corresponding citation (Yang* et al, *Nature 2008).*

3. In page 8, paragraph 2, the author stated that the gene expression dynamics during the induction of PGC precursors are developmentally conserved between the human and mouse species. This claim is not substantiated by the data provided by the authors.

Response 3: *In this revision, we emphasized the common and different gene expression of groups of genes that are expressed in human and mouse during the induction of precursors, and adjusted our claims accordingly (see also Fig.5E).*

4. In Figure 1C, a) the T/Hoechst image in the first raw of upper panel does not correspond to the other 3 images, judging from the pattern of nuclear staining. The authors should provide appropriate images, b) No BLIMP1 positive cells could be found in the day 1 image in the upper panel. Why? c) furthermore, the percentage of cells expressing T seems higher in day 2 than in day 1, which appears contradictory to the argument by the authors that the percentage of the cells expressing OCT4, BLIMP1 and T decreased in day 2 than in day 1, d) additionally, the color-combination in the lower panel should be changed because it is impossible to distinguish triple- from double-stained cells in the current version.

Response 4: a) *We corrected these mistakes and added a new panel of images.* b) *BLIMP1 expression was only moderately upregulated on day 1, which might reflect that the protein was not translated yet. c) We carefully reevaluated this point and revised Fig. 1C and D and the results and discussion sections accordingly. The percentage of cells expressing T did not change significantly on day 2, and OCT4$^+$/BLIMP1$^+$/T$^+$ cells emerged on day 2 only. d) We replaced the images and color combination to present the different cell populations better.*

5. Further cultivation of day 2 PGC precursors by BMP4 induces a fairly indistinct c-KIT(+)TRA-1-81(+) population, which appears to be largely overlapped with the c-KIT(-)TRA-1-81(intermediate) population. In particular, the upper right quadrant fraction (i.e., c-KIT(+)TRA-1-81(+) fraction) is essentially indistinguishable from lower right quadrant in day 6 of PGCLC induction (These fractions were used for the subsequent gene expression analysis). Accordingly, quadrants in Figure2B and C set by the authors appear somewhat arbitrary. Fluorescence intensity of the overall c-KIT(-) population appears fluctuating during the time course, which also raises the concern regarding the setting of the quadrant lines separating the positive and negative fractions. The authors need to address these points. The authors also need to show the negative control (isotype-matched control antibody staining) for each FACS data to justify the quadrant lines.

Response 5: *To confirm appropriate cKIT and TRA-1-81 stainings, we performed FACS first with unstained and isotope controls for positive and negative control cells. For the gating, we used the unstained controls as well as the isotype control. As requested, we added representative isotype controls to the figures (Fig. E2). The setting of the quadrant lines could have also been chosen to be slightly different; nevertheless, within time-course analysis spanning up to 8 days and analysis of different culture conditions, one can expect a certain fluctuation. We chose the presented settings to yield comparable fractions from different experiments.*

6. In Figure 2D, a) the authors should show the FACS plots of SSEA1/cKIT staining during the induction process and clarify the population sorted for the expression analysis, b) although the authors stated in the text "a very similar gene expression pattern", the data appear to indicate significantly lower expression of PGC markers in SSEA1/cKIT-positive cells than in TRA1-81/cKIT-positive cells. Why are they so different? The authors should analyze the induced cells by triple staining with cKIT, SSEA1 and TRA1-81, c) the data of pluripotency markers, such as OCT4, NANOG or SOX2 are not provided despite the statement that "we performed gene expression analysis for a subset of germ cell markers and pluripotency markers." Does TEX13B represent a germ cell marker? The expression levels should also ideally be presented as dCT value and the gene expression level needs to be compared between iPSCs, cKIT(+)TRA1-81(+) and cKIT(+)SSEA1(+) population.

Response 6: *a) We included the FACS plot of SSEA1/cKIT staining during the induction process in Fig. E2. b) We did not state that the fractions are identical; we tried to validate the cKIT/TRA-1-81 FACS strategy for PGCLC isolation, tested for specific germ cell markers, and observed similar expression data, i.e. expression of particular PGC-specific markers. Samples were compared to the iPSC level, and we did not expect them to be identical, as we sorted them in different combinations. However, the expression levels of BLIMP1, STELLA, and TEX13B are similar, and NANOS3 is also expressed in both populations, albeit at lower levels. c) We revised the sentence accordingly. d) TEX13B has been reported to be a specific germ cell marker (Sabour* et al, *HMG 2010).*

7. Figure 3B shows that only a subpopulation of c-KIT(+)TRA1-81(+) cells express BLIMP1. It is unclear why immune-staining of dissociated single cells from whole EBs explains the presence of BLIMP1-unstained cells while these cells are clearly positive for OCT4. This needs to be explained and the authors need to provide more quantitative data.

Response 7: *The FACS analysis of day 6 PGCLCs demonstrates a certain amount of TRA-1-81$^+$ cells, while the number of double-positive cells decreases, which could explain the presence of OCT4$^+$/BLIMP1$^-$ cells.*

8. Figure 3D is the only data supporting the global DNA demethylation in PGCLCs, therefore needs to be carefully validated. The immunostaining of 5mC on iPSCs and PGCLCs needs to be done either side-by-side in the same slide or with the same positive control in each slide. Also, the anti-5mC antibody is the mouse monoclonal IgG antibody (amm99021; Aviva), thus the secondary antibody used for the immunostaining protocol could react with both anti-5mC primary antibody and anti-c-KIT primary antibody (mouse IgG) used for the sorting, which potentially prevents the specific recognition of 5mC expression.

Response 8: *This referee raises a valid technical concern. Indeed, the dual reactivity of the secondary antibody and the subsequent prevention of specific recognition of 5mC could be an issue. However, we observed a clear reduction in 5mC staining, which argues against this concern. If the secondary antibody had reacted with the anti-c-KIT primary antibody, we would have observed an overall staining of all the cells, which was not the case. It is also unlikely that the cKIT antibody did resist the HCl treatment before the 5mC staining. We also included another cKIT$^+$ cell population as a control in the experiment. If the mouse IgG would have reacted with the cKIT antibody, the positive signal would have been observed only at the plasma membrane, which was not the case.*

9. In Figure 3E(iv), the authors need to provide images with higher resolution and/or magnification, since it is unclear whether the pattern of NUMA staining is specific or not. Please also provide the scales in Figure 3E (iii) and (iv).

Response 9: *The NUMA staining is specific, as the majority of cells in direct vicinity to the NUMA$^+$ cells are clearly NUMA$^-$. We purposely chose this magnification to demonstrate the dispersed occurrence of NUMA$^+$ cells. We now inserted a higher magnification image of one region. We also added the scale bars.*

10. In Figure 4B, the edge of the contour plots extends out of the frame. PMT voltage or gains needs to be adjusted appropriately.

Response 10: *We kept the PMT voltage on the Tra1-81 channel and all other channels constant, although a small proportion of cells (fewer than 3% in all instances) appeared on the right edge of the plot. We wanted to make sure that all samples were measured with the same amplification settings, thereby ensuring the comparability of the different series of experiments. Importantly, the appearance of cells on the edges of the plot does not change the statistics.*

11. In Figure 5C, the expression levels of OCT4 in both PGCLCs and iPSCs are markedly low compared with other pluripotency markers. This raises a serious concern on the pluripotency of the iPSC line used in the study. This needs to be explained.

Response 11: *The chip has several probes for one gene, and unfortunately the probe initially chosen to automatically target OCT4 expression, exhibited mechanically low binding in all samples. We have corrected this error in the new version, where all the genes are represented by the most responsibe probes now. OCT4 is now higher expressed in iPSC. This is also evident from the heat map that has been included in Fig. 5E. In addition, there is no doubt about the pluripotent state of the iPSC line used in this study, as the same line generated teratomas in the ovarian transplantation experiment shown.*

Material and Methods:

In page 23, first paragraph, the sentence "For generation of stable GFP reporter lines...." needs to be removed.

Response: *We removed it.*

In page 24, second paragraph, the information regarding anti-c-KIT antibody (550412; eBioscience) is likely incorrect.

Response: *This is correct. The antibody we used was 17-1179.*

In page 24, third paragraph, authors need to provide the more detailed information including the methods of attachment of the cells to microslides and conjugated fluorochromes of the secondary antibodies.

Response: *We added the requested information in the Methods.*

In page 29, second paragraph, authors need to provide the more detailed protocol regarding the transfection of shRNA, such as the amount of the shRNA, cell numbers, or the timing when the cells were used for the assay.

Response: *We transfected d2 precursor cultures with shRNA and evaluated d4 PGCLCs. We supplemented the Methods section with more detailed information.*

Referee #3:

I have few further comments on the revised manuscript for the authors to consider:

1.The authors highlight a potentially important observation on Prdm14 that shows modest expression in human PGC-like cells (not the case in mice), which is emphasized in the title and the abstract. Since Prdm14 antibodies are commercially available and have previously been used in some studies on human ES cells, it would add significantly to their assertions to have immunofluorescence data for Prdm14 on ESCs, precursors and PGC-like cells. The Prdm14 knockdown study which has no effect on PGC-like cells (a negative result and therefore requires caution), would also be greatly helped by Western blot analysis to show if there is any protein there at all, and to see the effectiveness of the knockdown. This would provide data to support their conclusions.

Response 1: *We now included immunofluorescence data for* PRDM14 *on d0 and d2 PGC precursor induction cultures (Fig. E11), and observed a significant signal reduction by day 2.*

3. Page 8: The yield of PGC-like cells from different lines is stated to vary and was as low as 5% depending on the cell line. It would be generally useful to know how many lines were tested and what was the efficiency in each case.

Response 2: *We included PGCLC derivation from HuES6 and SA8 iPSCs in Fig. E3. On average, we obtained ca. 10% PGCLCs from hESCs and 10–20% from iPSCs. Yields were significantly lower when low-quality PSC cultures were used as starting materials.*

