Germline development in rat revealed by visualization and deletion of *Prdm14*

Toshihiro Kobayashi, Hisato Kobayashi, Teppei Goto, Tomoya Takashima, Mami Oikawa, Hiroki Ikeda, Reiko Terada, Fumika Yoshida, Makoto Sanbo, Hiromitsu Nakauchi, Kazuki Kurimoto and Masumi Hirabayashi
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I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

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

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

Reviewer 1

Advance summary and potential significance to field

In this report, Kobayashi and co-workers investigate PGC development in rats as a means to identify similarities and differences between rodent species as well as other mammals. The motivation for this work is to provide a novel resource in the form of a PGC reporter rat line that enables tracking of PGC development in this species that has been shown to exhibit multiple properties more similar to humans, compared with mice. The authors use this line to track the progression of PGC development, which has not previously been attempted. By crossing heterozygotes with an alternative Prdm14 KO line without the reporter they are able to investigate the requirement for Prdm14 during rat germ cell specification and development. Surprisingly, they find that PGC development fails completely, with failure to initiate expression of Tfap2c, so that there are no germ cells in the gonads post-natally, leading to overt reduction in the size of the gonads in both males and females. This contrasts with the phenotype in the mouse, in which PGCs appear to be specified (Tfap2c positive) but subsequently fail to develop normally. Finally, the authors perform RNAseq using cells sorted for Prdm14-H2BVenus from various key stages of germ cell development, thus providing the first resource for comparison with epiblast, PGCs and sex-determined germ cells from mice and other species. Their analysis uncovers large similarities with mouse germ cells at similar stages with a few distinctions, reflecting the potential more stringent requirement for Prdm14 in rats compared with mice implicated from their analysis of KO embryos. This is a very well planned and expertly executed undertaking that confirms several expected roles, but also uncovers a few novel rat-specific developmental details.

Minor points to be addressed:

1. The images of Prdm14-H2BVenus fluorescence for E7.5, 8.5, 9.5 and 10.5 whole mounts in Figure 1B are not of sufficient magnification/resolution for the reader to distinguish whether there is any fluorescence (the dashed line does not help, but rather obscures the area). Since in figure 2 the confocal images are so clear these panels may be dispensable from Figure 1B?

2. P6, line 16 and last sentence are quite misleading because they imply that Prdm14 is essential for epiblast specification in both mice and rats, but this would result in early postimplantation lethality, which is not the case in either species. Data available so far indicate that Prdm14 enhances epiblast formation, rather than drives it in vivo. Please rewrite the sentence to reflect this.

3. P6, line 6 and P8 line 4: The word ‘homogenous’ should be replaced with ‘homogeneous’.

4. P8, line 13: ‘Fig 1A’ should be replaced by ‘Fig 1B’ (unless this panel is removed, as suggested above).

5. P9, line 17: please provide reference for the transient expression of Sox17 observed in early stages of PGC specification in mice.

6. P12, line 16: please provide a reference to support the statement regarding similarity of the factors identified to be involved in rat PGC development and maintenance compared with mice and other species.

7. The distinction between rat and mouse PGC specification in Prdm14 null embryos in the context of Tfap2c activation merits mention in the abstract, particularly since there is space.

Reviewer 2

Advance summary and potential significance to field

This manuscript described a detailed process of rat primordial germ cell (PGC) development by using Prdm14-Venus knock-in rat, and roles of PRDM14 in rat PGC development. The authors concluded that the developmental process of PGCs and functional importance of PRDM14 for PGC

specification were conserved well between mouse and rat, though they suggested more stringent regulation of PGC formation by PRDM14 in rat compared with that in mouse.

All experiments have been nicely performed, and comprehensive data concerning rat PGC development is beautifully presented, while a main conclusion is a conserved PGC development in mouse and rat, which is not surprising. More in-depth description concerning any differences of mouse and rat PGC development likely improves this manuscript.

Comments for the author

1. Comparison of PGC-specific gene/protein expression and of abnormalities of Pdrm14-deficient PGCs in early embryos between rat and mouse are often unclear and confusing. More careful comparison based on consistent morphological criteria instead of embryonic day in the text corresponding to Fig.2, 3, S3 may be helpful. In addition, the expression of Blimp1 and Tfap2c in rat embryos earlier than LSOB (E8.5) may be informative to compare mouse and rat PGC specification.

2. The authors discussed possible differences of mouse and rat PRDM14 functions in PGC specification based on the immune-staining data in Fig.3A,B (page 10, lines 16-21; page 13, line 24). Because published mouse data showed the expression of the markers at gene expression level, it is necessary to show the decreased expression of Blimp1 etc. in Prdm14-deficient pre-migratory rat PGCs by PCR to compare rat with mouse.

3. Page 11, lines 17-24; Fig.4A, S5D; Although the authors discussed that the PC2/PC3 scatter plot reflect a temporal trajectory from epiblast to PGCs, contribution of PC2/PC3 is small compared with that of PC1, suggesting that transcriptional changes do not necessarily reflect a temporal trajectory. More careful discussion may be appropriate.

4. Page 13, lines 4-15; Fig.S6B; The authors discussed that the expression patterns of genes in each cluster in mouse and rat is relatively similar. However, Fig.S6B dose not necessarily suggest similarity, but may imply the overall differences of gene expression changes between rat and mouse PGCs. Extraction of differentially expressed genes and discussion of the meanings of the differential expression may be informative to think about possible differences of rat and mouse PGC development.

5. Citation of Figures in the text may be sometimes wrong (page 8, Fig.3; page 13, Fig.S5).

Reviewer 3

Advance summary and potential significance to field

It was a pleasure to read this interesting manuscript by Kobayashi and colleagues. They generate a Prdm14 reporter rat model and use this to provide the first detailed analysis of rat PGC development – including immunostaining and RNA-seq analysis. They combine this allele with an independently generated knockout allele and demonstrated that Prdm14 is a critical regulator of PGC development in the rat, as has previously been reported in the mouse and more recently, in humans (bioRxiv 563072).

In addition to being the first detailed description of PGC development in rat, this represents the first targeted allele with a germ line phenotype in this species (to my knowledge) and also generates a new reporter allele which will benefit future studies of rat PGC development. Since the derivation of rat ES cells in 2008, there have been relatively few studies which have utilised transgenic approaches such as this to address developmental questions, and as such this study has extra significance and should give confidence to others in the field. While it might have been predicted that key regulators of PGC development would be conserved between mice and rats, this is the first study to formally address this, and therefore represents an important addition in the field.

While I do have some comments that should be addressed, I think this study has all the makings of a very strong candidate for publication in Development.

Comments for the author

Major comments:

1. Introduction. The introduction could be improved by being more accurate/specific in the language used. The paragraph starting on Page 3 line 21 is most troublesome, as the authors seek to highlight advances in human germ cell research and needlessly over-emphasise the differences with mouse. In particular:

a) 'Use of surrogate models such as pig and monkey have revealed critical differences in genetic programs between and in the origin of PGCs between mouse and human'. This statement is very strange – how do studies in monkeys and pigs have any bearing on similarities or differences between mouse and humans?

There appear to be quite significant differences in the origins of PGCs between monkeys and humans – why are these ignored here? We have not even started to examine the differences within non-human primate models. In addition, there are some clear differences in development and gene expression between pigs and humans also. To complicate matters further there are rodents with a bilaminar disc that are divergent from pig, monkey and human, suggesting that similarities in embryology are not key to understanding gene expression programs. (Lagostomus maximus has a bilaminar disc and PGCs do not express Sox2 (like human/pig), however Sox17 expression occurs later and Blimp1 is not expressed at all (Leopardo and Vitullo, 2017)).

It is simply too early to make generalizations regarding differences between phylla and which features might exhibit species-specificity. Indeed, a major strength of this manuscript is that it adds a detailed description of another species for comparison. It seems the authors are attempting to discredit the very important work that has been done in the mouse, which has laid the foundation for the entire field – including this paper. In my view, this study is strong enough in its own right without resorting to this kind of point scoring.

b) Page 4 Line 1: 'critical differences' – this is a very subjective term. Which differences do the authors think are critical? If they wish to make this point, then it should be balanced by the many critical similarities (which is a much longer list). Alternatively, this statement could be altered or removed.

c) Page 4 line 3. 'This species-wide difference in PGCs' – again it isn't exactly clear what difference the authors are referring to here?

2. Early gene expression profiles (Figure 4C). Greater explanation of the differences between Cluster and 5 and 6 is needed. It appears that genes in Cluster 5 are upregulated earlier (and more strongly) and then maintained in rat germ cells, whereas cluster 6 is upregulated later (and less strongly) and then decrease at E15.5. It is surprising to see both Prdm1 and Prdm14 in Cluster 6 – especially as the Prdm14 reporter is highly expressed at all PGC stages in this RNA-seq analysis. Properly explaining these groups, making very clear the expression changes in key genes and how they relate to each other, and then critically assessing how they compare with the mouse would improve the manuscript.

3. Nanog. From Figure 4C it appears that Nanog is rapidly upregulated and maintained in rat PGCs. It would be very interesting if the authors could show immunostainings from E7.25 – E9.0 for Nanog (similar to Figure 2)– to see exactly how Nanog expression changes during PGC specification in rats. This is important as the role in Nanog in mouse PGCs is somewhat unclear – Nanog was originally suggested to be downregulated in early mouse PGC (Hatano et al., 2005; Yamaguchi et al., 2005). More recently, overexpression has been reported to promote PGC specification in vitro (Murakami et al., 2016), while at the same time Nanog has been shown to be non-essential for PGC development (Zhang et al., 2018b). In addition, Nanog appears to be a very early marker of PGCs in the pig (Kobayashi et al., 2017). Therefore verifying the role of Nanog during PGC specification in rats would contribute to cross-species understanding of Nanog's role in the germ line.

4. Cross species comparative analysis. This could be improved. Although the overall pattern of correlation does seem to hold in Figure 4E (particularly with the Yamashiro et al. 2015 data), the correlation co-efficients are very low – why is this? In Figure S6A the dendrogram appears to simply show that mouse PGCs cluster separately to rat – the three main stems are 'late' rat PGCs (E10.5 – E15.5), early rat PGCs and then mouse. Is it surprising that the E13.5 mouse PGCs cluster closer to the early rat PGC samples – and quite distinct to the late rat samples? Also, since the authors mention the importance of understanding human PGC development in their introduction, I was surprised that a broader cross species comparison was not attempted – to include humans and any other species in which appropriate data is available. This should be quite straightforward given the large number of published human PGC RNA-seq datasets now available. A broader comparison would provide an unbiased way to assess whether rat PGC development really does more closely mirror that of mouse.

5. Prdm14 knock out phenotype. What happens to the Prdm14 KO PGCs? Do they die by apoptosis? Or differentiate? If this is difficult to show directly in the embryo, then it would be possible to sort the knockout PGCs using the Venus reporter and perform RNA-seq/qPCR. This would allow interesting cross-comparison with expression data available for Prdm14 KO PGCs in mice (Grabole et al., 2013; Yamaji et al., 2008) and human (bioRxiv 563072), and would be a really valuable addition to this manuscript.

Minor issues.
1. Page 5, Line 7. The Hajkova lab also made a significant contribution to the role of Prdm14 in acquisition of naïve pluripotency and in driving hypomethylation (Leitch et al., 2013).

2. Page 5 line 15. The study by Northrup et al., 2011 includes RT-PCR analysis of a number of genes in rat genital ridges and also suggests conserved function of Dnd1 in this species.

3. Prdm14-H2BVenus knockin ES cells. A number of other details appear to be missing. Which parental ES cell line was used, and has this previously been published? What strain and sex is this ES cell line? Southern blots should be shown in the supplementary material to verify the successful homologous recombination and rule out off target effects.

4. Prdm14-H2BVenus mice: How many generations have these heterozygous mice been bred for? Can homozygous mice be obtained and do they phenocopy the Prdm14mut/HV mice?

5. T/Brachyury. The relationship between Prdm14-H2B Venus should be made clearer. During Day 8, do all Prdm14 reporter positive cells emerge from T-positive cells? Perhaps an attempt to quantify the immunostainings in Figure 2 would make things clearer (i.e. what percentage of Prdm14-positive cells are T positive at each stage shown). It looks from the images presented that many Venus positive cells are not T positive – in keeping with the finding that T is non-essential for PGC development (Zhang et al., 2018a)

6. Figure 2 at E8.75 there are a large number of Venus positive cells that are quite distant to the main cluster, including some that are very anterior. Are these PGCs that have been specified ectopically? Or have they migrated away? Would staining for another marker be useful to identify what these cells are?

7. Figure S3B – the correlation between Sox2 and Venus is very strong at E9.5, but there appears to be a number of PGCs with low/absent Oct4 – in particular those cells which have migrated furthest from the allantois. Is this really the case?

8. Prdm14mut/+ mice: No data is shown to verify the mutation in these mice – please include this in supplementary material. Can this mouse be readily maintained as a heterozygous breeding colony?

9. Page 9, line 13. 're-acquisition of pluripotency'. This term appears to be used as a direct carry-ver from descriptions often used in the mouse. However, other than Sox2, which pluripotency genes are actually turned off and re-expressed during rat PGC specification, and which genes are maintained? (Although Oct4, levels do decrease somewhat it is known in mouse that a 50%

reduction in Oct4 actually improves self-renewal of mouse ES cells (Karwacki-Neisius et al., 2013) and so this is unlikely to influence the 'pluripotent character' of rat PGCs.) A great benefit of this new and exciting rat data is to really question which aspects are conserved with mice, and so the authors should be cautious before adopting the principles which have become accepted dogma in mice.

10. Page 10, Line 19. The study by the Surani laboratory also contributed to the findings described here (Grabole et al., 2013)

11. Page 14, line 2. The study by the Ying laboratory should be included here (Li et al., 2008)

12. The recent studies from the Payer lab (Mallol et al., 2019) and Surani lab (bioRxiv 563072) should be discussed and cited.

References:

Grabole, N., Tischler, J., Hackett, J.A., Kim, S., Tang, F., Leitch, H.G., Magnúsdóttir, E., and Surani, M.A. (2013). Prdm14 promotes germline fate and naive pluripotency by repressing FGF signalling and DNA methylation. EMBO Rep 14, 629–637.

Hatano, S.-Y., Tada, M., Kimura, H., Yamaguchi, S., Kono, T., Nakano, T., Suemori, H., Nakatsuji, N., and Tada, T. (2005). Pluripotential competence of cells associated with Nanog activity. Mech Dev 122, 67–79.

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Leopardo, N.P., and Vitullo, A.D. (2017). Early embryonic development and spatiotemporal localization of mammalian primordial germ cell-associated proteins in the basal rodent Lagostomus maximus. Sci. Rep. 7, 594–11.

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Yamaguchi, S., Kimura, H., Tada, M., Nakatsuji, N., and Tada, T. (2005). Nanog expression in mouse germ cell development. Gene Expr Patterns 5, 639–646.

Yamaji, M., Seki, Y., Kurimoto, K., Yabuta, Y., Yuasa, M., Shigeta, M., Yamanaka, K., Ohinata, Y., and Saitou, M. (2008). Critical function of Prdm14 for the establishment of the germ cell lineage in mice. Nat Genet 40, 1016–1022.

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Zhang, M., Leitch, H.G., Tang, W.W.C., Festuccia, N., Hall-Ponsele, E., Nichols, J., Surani, M.A., Smith, A., and Chambers, I. (2018b). Esrrb Complementation Rescues Development of Nanog-Null Germ Cells. Cell Reports 22, 332–339.

First revision

Author response to reviewers' comments

We thank you all for your time and effort in reviewing our manuscript DEVELOP/2019/183798 entitled " Germline development in rat revealed by visualization and deletion of Prdm14". We have done our best to address all the concerns raised and have incorporated valuable suggestions by the reviewers. This has strengthened and improved the manuscript. Here is a point-wise response to the reviewer's comments.

Reviewer 1
Advance summary and potential significance to field

In this report, Kobayashi and co-workers investigate PGC development in rats as a means to identify similarities and differences between rodent species as well as other mammals. The motivation for this work is to provide a novel resource in the form of a PGC reporter rat line that enables tracking of PGC development in this species that has been shown to exhibit multiple properties more similar to humans, compared with mice. The authors use this line to track the progression of PGC development, which has not previously been attempted. By crossing heterozygotes with an alternative Prdm14 KO line without the reporter they are able to investigate the requirement for Prdm14 during rat germ cell specification and development.

Surprisingly, they find that PGC development fails completely, with failure to initiate expression of Tfap2c, so that there are no germ cells in the gonads post-natally, leading to overt reduction in the size of the gonads in both males and females. This contrasts with the phenotype in the mouse, in which PGCs appear to be specified (Tfap2c positive) but subsequently fail to develop normally. Finally, the authors perform RNAseq using cells sorted for Prdm14-H2BVenus from various key stages of germ cell development, thus providing the first resource for comparison with epiblast, PGCs and sex-determined germ cells from mice and other species. Their analysis uncovers large similarities with mouse germ cells at similar stages with a few distinctions, reflecting the potential more stringent requirement for Prdm14 in rats compared with mice implicated from their analysis of KO embryos. This is a very well planned and expertly executed undertaking that confirms several expected roles, but also uncovers a few novel rat-specific developmental details.

We thank the reviewer for the encouraging, supportive and constructive comments.

Reviewer 1 Comments for the author

Minor points to be addressed:

1. The images of Prdm14-H2BVenus fluorescence for E7.5, 8.5, 9.5 and 10.5 whole mounts in Figure 1B are not of sufficient magnification/resolution for the reader to distinguish whether there is any

fluorescence (the dashed line does not help, but rather obscures the area). Since in figure 2 the confocal images are so clear these panels may be dispensable from Figure 1B?

We thank the reviewer for pointing this out. In the revised Fig.1B, we have replaced the E7.5, 8.5, 9.5 and 10.5 embryos whole mounts with clearer images.

2. P6, line 16 and last sentence are quite misleading because they imply that Prdm14 is essential for epiblast specification in both mice and rats, but this would result in early postimplantation lethality, which is not the case in either species. Data available so far indicate that Prdm14 enhances epiblast formation, rather than drives it in vivo. Please rewrite the sentence to reflect this.

We agree with the reviewer's suggestion, and have modified the sentence, accordingly (P6 line 17).

3. P6, line 6 and P8 line 4: The word 'homogenous' should be replaced with 'homogeneous'.

We have fixed the misspelling.

4. P8, line 13: 'Fig 1A' should be replaced by 'Fig 1B' (unless this panel is removed, as suggested above).

We have corrected the figure number.

5. P9, line 17: please provide reference for the transient expression of Sox17 observed in early stages of PGC specification in mice.

We have added in a new reference (Yabuta et al., 2006), which shows transient upregulation of Sox17 in mouse PGCs by single cell RT-qPCR.

6. P12, line 16: please provide a reference to support the statement regarding similarity of the factors identified to be involved in rat PGC development and maintenance compared with mice and other species.

We have added in 2 recent references reviewing the role of transcription factors in mammalian PGC development and maintenance (Bleckwehl and Rada-Iglesias, 2019; Sybirna et al., 2019).

7. The distinction between rat and mouse PGC specification in Prdm14 null embryos in the context of Tfap2c activation merits mention in the abstract, particularly since there is space.

According to the reviewer's suggestion, we have modified the abstract and added in a sentence to that effect (P2 lines 10-12).

Reviewer 2

Advance summary and potential significance to field

This manuscript described a detailed process of rat primordial germ cell (PGC) development by using Prdm14-Venus knock-in rat, and roles of PRDM14 in rat PGC development. The authors concluded that the developmental process of PGCs and functional importance of PRDM14 for PGC specification were conserved well between mouse and rat, though they suggested more stringent regulation of PGC formation by PRDM14 in rat compared with that in mouse. All experiments have been nicely performed, and comprehensive data concerning rat PGC development is beautifully presented, while a main conclusion is a conserved PGC development in mouse and rat, which is not surprising. More in-depth description concerning any differences of mouse and rat PGC development likely improves this manuscript.

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Reviewer 2 Comments for the author

1. Comparison of PGC-specific gene/protein expression and of abnormalities of Pdrm14-deficient PGCs in early embryos between rat and mouse are often unclear and confusing. More careful comparison based on consistent morphological criteria instead of embryonic day in the text corresponding to Fig.2, 3, S3 may be helpful. In addition, the expression of Blimp1 and Tfap2c in rat embryos earlier than LSOB (E8.5) may be informative to compare mouse and rat PGC specification.

According to reviewer's suggestion, in the text, we have added morphological criteria based on Fig.S3 to the analyzed embryos.

Regarding the expression of Blimp1 and Tfap2c in earlier stages of rat embryos, we checked their expression in E8.0 rat embryos. While Tfap2c is expressed in the extraembryonic ectoderm as in E8.5 (revised Fig. 2C), we could not detect any Blimp1 positive cells then. Since staining by Blimp1 antibody is not as sensitive as Tfap2c, we have not concluded yet whether it is completely absent or not. In mice, the sensitive reporters (Blimp1-EGFP, or Blimp1-Venus) have been used to track initiation of Blimp1 expression. Further study using such reporters in rats would be important to reveal its precise expression pattern. Thus, in this paper, we focus on Blimp1 and Tfap2c staining at E8.5 and E9.5.

2. The authors discussed possible differences of mouse and rat PRDM14 functions in PGC specification based on the immune-staining data in Fig.3A,B (page 10, lines 16-21; page 13, line 24). Because published mouse data showed the expression of the markers at gene expression level, it is necessary to show the decreased expression of Blimp1 etc. in Prdm14-deficient pre-migratory rat PGCs by PCR to compare rat with mouse.

To reveal the difference of Prdm14 function between mouse and rat, we have performed single cell RT-qPCR analysis of rat PGCs collected from Prdm14 heterozygous or homozygous embryos. The data in revised Fig.3A clearly shows the impairment in Prdm14 KO PGCs in activating germ cell markers, pluripotency genes as well as genes related to DNA demethylation, which are in part similar to mouse but show some differences (discussed in P10 lines 14-21).

3. Page 11, lines 17-24; Fig.4A, S5D; Although the authors discussed that the PC2/PC3 scatter plot reflect a temporal trajectory from epiblast to PGCs, contribution of PC2/PC3 is small compared with that of PC1, suggesting that transcriptional changes do not necessarily reflect a temporal trajectory. More careful discussion may be appropriate.

In response to the reviewer's insightful comment, we have carefully re-analyzed the data and found that PC1 reflects significant differential expression of genes related to mitochondrial function (revised Fig.S5G). Since the number of mitochondria change dramatically from PGC specification to subsequent development (Cree et al., 2008; Floros et al., 2018), the expression of mitochondrial genes might largely influence the transcriptome in rats as well. We have added the data and a sentence with the citations in the revised manuscript (P11 lines 17-21).

4. Page 13, lines 4-15; Fig.S6B; The authors discussed that the expression patterns of genes in each cluster in mouse and rat is relatively similar. However, Fig.S6B dose not necessarily suggest similarity, but may imply the overall differences of gene expression changes between rat and mouse PGCs. Extraction of differentially expressed genes and discussion of the meanings of the differential expression may be informative to think about possible differences of rat and mouse PGC development.

To prevent an effect due to technical differences, we have replaced mouse data with new data, obtained by a similar protocol and have looked at representative genes known to function in mouse PGCs (revised Fig.4D, S6A,B). Furthermore, we also included human PGC data for comparison (Tang et al., 2015). The new comparisons highlight highly conserved expression patterns within rodents but not with human (revised Fig.4D, S6A,B). We have added the data and discussed it in the revised manuscript (P12 line 21 - P13 line 11).

5. Citation of Figures in the text may be sometimes wrong (page 8, Fig.3; page 13, Fig.S5).

We have carefully checked all the citation of Figures and fixed them.

Reviewer 3

Advance summary and potential significance to field

It was a pleasure to read this interesting manuscript by Kobayashi and colleagues. They generate a Prdm14 reporter rat model and use this to provide the first detailed analysis of rat PGC development – including immunostaining and RNA-seq analysis. They combine this allele with an independently generated knockout allele and demonstrated that Prdm14 is a critical regulator of PGC development in the rat, as has previously been reported in the mouse and more recently, in humans (bioRxiv 563072). In addition to being the first detailed description of PGC development in rat, this represents the first targeted allele with a germ line phenotype in this species (to my knowledge) and also generates a new reporter allele which will benefit future studies of rat PGC development. Since the derivation of rat ES cells in 2008, there have been relatively few studies which have utilised transgenic approaches such as this to address developmental questions, and as such this study has extra significance and should give confidence to others in the field. While it might have been predicted that key regulators of PGC development would be conserved between mice and rats, this is the first study to formally address this, and therefore represents an important addition in the field.

While I do have some comments that should be addressed, I think this study has all the makings of a very strong candidate for publication in Development.

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Reviewer 3 Comments for the author

Major comments:

1. Introduction. The introduction could be improved by being more accurate/specific in the language used. The paragraph starting on Page 3 line 21 is most troublesome, as the authors seek to highlight advances in human germ cell research and needlessly over-emphasise the differences with mouse. In particular:

a) ‘Use of surrogate models such as pig and monkey have revealed critical differences in genetic programs between and in the origin of PGCs between mouse and human’. This statement is very strange – how do studies in monkeys and pigs have any bearing on similarities or differences between mouse and humans? There appear to be quite significant differences in the origins of PGCs between monkeys and humans – why are these ignored here? We have not even started to examine the differences within non-human primate models. In addition, there are some clear differences in development and gene expression between pigs and humans also. To complicate matters further there are rodents with a bilaminar disc that are divergent from pig, monkey and human, suggesting that similarities in embryology are not key to understanding gene expression programs. (Lagostomus maximus has a bilaminar disc and PGCs do not express Sox2 (like human/pig), however Sox17 expression occurs later and Blimp1 is not expressed at all (Leopardo and Vitullo, 2017)). It is simply too early to make generalizations regarding differences between phylla and which features might exhibit species-specificity. Indeed, a major strength of this manuscript is that it adds a detailed description of another species for comparison. It seems the authors are attempting to discredit the very important work that has been done in the mouse, which has laid the foundation for the entire field – including this paper. In my view, this study is strong enough in its own right without resorting to this kind of point scoring.

b) Page 4 Line 1: ‘critical differences’ – this is a very subjective term. Which differences do the authors think are critical? If they wish to make this point, then it should be balanced by the many critical similarities (which is a much longer list). Alternatively, this statement could be altered or removed.

c) Page 4 line 3. ‘This species-wide difference in PGCs’ – again it isn’t exactly clear what difference the authors are referring to here?

To address the reviewer’s comment, we have decided to remove the paragraph and not over-emphasize species wide differences beyond published data, and focus on the mouse model system.

2. Early gene expression profiles (Figure 4C). Greater explanation of the differences between Cluster and 5 and 6 is needed. It appears that genes in Cluster 5 are upregulated earlier (and more strongly) and then maintained in rat germ cells, whereas cluster 6 is upregulated later (and less strongly) and then decrease at E15.5. It is surprising to see both Prdm1 and Prdm14 in Cluster 6 – especially as the Prdm14 reporter is highly expressed at all PGC stages in this RNA-seq analysis. Properly explaining these groups, making very clear the expression changes in key genes and how they relate to each other, and then critically assessing how they compare with the mouse would improve the manuscript.

While K-means analysis automatically assigned distinct clusters 5 and 6, overall these clusters contain germ cell-related genes. The difference lies in the expression level of the genes, Fig.4D shows relatively lower expression of Prdm1 and Prdm14 in cluster 6 than Tfap2c, Nanos3 and Dnd1 in cluster 5. We have tweaked the sentence (P12 line 12-13).Slight reduction of germ cell related genes is also observed in revised Fig.4C which is also observed in mouse (revised Fig.4D), suggesting the reduction might be associated with onset of sex-specific differentiation and meiosis as recently shown (Hill et al., 2018).

3. Nanog. From Figure 4C it appears that Nanog is rapidly upregulated and maintained in rat PGCs. It would be very interesting if the authors could show immunostainings from E7.25 – E9.0 for Nanog (similar to Figure 2) – to see exactly how Nanog expression changes during PGC specification in rats. This is important as the role in Nanog in mouse PGCs is somewhat unclear – Nanog was originally suggested to be downregulated in early mouse PGC (Hatano et al., 2005; Yamaguchi et al., 2005). More recently, overexpression has been reported to promote PGC specification in vitro (Murakami et al., 2016), while at the same time Nanog has been shown to be non-essential for PGC development (Zhang et al., 2018b). In addition, Nanog appears to be a very early marker of PGCs in the pig (Kobayashi et al., 2017). Therefore verifying the role of Nanog during PGC specification in rats would contribute to cross-species understanding of Nanog’s role in the germ line.

We certainly agree on the importance of looking at Nanog expression pattern during rat PGC specification. However, unfortunately, a good quality Nanog antibody to stain whole mount or cryosections is not yet commercially available. We have tested several antibodies from Abcam, Santa cruz, Cosmobio and Peprotech (some of them work in cultured cells), but failed to obtain clear signals for embryonic sections. Since, the amino acid sequence of Nanog is relatively diverse across species (Theunissen et al., 2011), antibodies which recognize mouse or human Nanog may not readily work for rat, particularly for in vivo samples. Thus, raising a novel antibody against rat Nanog or generating a new knock-in reporter into the Nanog locus would be necessary to trace its expression pattern. Further experiments using an in vitro system, which has not yet been achieved in rat, would reveal the functional conservation and diversity among the species. We believe it is definitely an interesting point but beyond the purview of this paper, and we would like to address this aspect in a future study.

4. Cross species comparative analysis. This could be improved. Although the overall pattern of correlation does seem to hold in Figure 4E (particularly with the Yamashiro et al. 2015 data), the correlation co-efficients are very low – why is this? In Figure S6A the dendrogram appears to simply show that mouse PGCs cluster separately to rat – the three main stems are ‘late’ rat PGCs (E10.5 – E15.5), early rat PGCs and then mouse. Is it surprising that the E13.5 mouse PGCs cluster closer to the early rat PGC samples – and quite distinct to the late rat samples? Also, since the authors mention the importance of understanding human PGC development in their introduction, I was surprised that a broader cross species comparison was not attempted – to include humans and any other species in which appropriate data is available. This should be quite straightforward given the large number of published human PGC RNA-seq datasets now available. A broader comparison would provide an unbiased way to assess whether rat PGC development really does more closely mirror that of mouse.

According to the reviewer's suggestion, we have incorporated a published human PGC data from the Surani lab and compared the expression of key PGC regulators as shown in the previous report (Tang et al., 2015). The data now shown in revised Fig.4D clearly exhibits similarity within rodents and some difference with human, in particular, expression of SOX2, SOX17 and naïve pluripotency gene such as KLF4, TFCP2L1. We have discussed the data in the revised manuscript (P13 lines 9-11).

5. Prdm14 knock out phenotype. What happens to the Prdm14 KO PGCs? Do they die by apoptosis? Or differentiate? If this is difficult to show directly in the embryo, then it would be possible to sort the knockout PGCs using the Venus reporter and perform RNA-seq/qPCR. This would allow interesting cross-comparison with expression data available for Prdm14 KO PGCs in mice (Grabole et al., 2013; Yamaji et al., 2008) and human (bioRxiv 563072), and would be a really valuable addition to this manuscript.

To reveal the phenotype of Prdm14 KO rat PGCs, we have performed single cell RT-qPCR analysis. The data in the revised Fig.3A clearly shows impairment of activating germ cell markers, pluripotency genes as well as genes related to DNA methylation in Prdm14 KO PGCs, which are in part similar to mouse but show some differences (discussed in P10 lines 14-21).

Minor issues.

1. Page 5, Line 7. The Hajkova lab also made a significant contribution to the role of Prdm14 in acquisition of naïve pluripotency and in driving hypomethylation (Leitch et al., 2013).

We have added it in the revised manuscript.

2. Page 5 line 15. The study by Northrup et al., 2011 includes RT-PCR analysis of a number of genes in rat genital ridges and also suggests conserved function of Dnd1 in this species.

We have added this in the revised manuscript.

3. Prdm14-H2BVenus knockin ES cells. A number of other details appear to be missing. Which parental ES cell line was used, and has this previously been published? What strain and sex is this ES cell line? Southern blots should be shown in the supplementary material to verify the successful homologous recombination and rule out off target effects.

We have added details pertaining to Prdm14-H2BVenus knockin ES cells in Materials and methods (P18 lines 8-11) and in revised Fig.SA,B.

4. Prdm14-H2BVenus mice: How many generations have these heterozygous mice been bred for? Can homozygous mice be obtained and do they phenocopy the Prdm14mut/HV mice?

So far, we have crossed Prdm14-H2BVenus rats for more than 4 generations and all the generations show consistent phenotypes in terms of reporter expression in heterozygous mutants and lack of germ cells in homozygous mutants. We have not checked Prdm14HV/HV homozygous rats, but have been using either male or female Prdm14HV/+ rats to obtain Prdm14HV/mut. Since both combinations show identical phenotypes, the homozygous KO will have the same phenotype.

5. T/Brachyury. The relationship between Prdm14-H2B Venus should be made clearer. During Day 8, do all Prdm14 reporter positive cells emerge from T-positive cells? Perhaps an attempt to quantify the immunostainings in Figure 2 would make things clearer (i.e. what percentage of Prdm14-positive cells are T positive at each stage shown). It looks from the images presented that many Venus positive cells are not T positive – in keeping with the finding that T is non-essential for PGC development (Zhang et al., 2018a)

We have quantified the number of T positive cells in Prdm14-H2BVenus positive PGCs at E8.5 and E8.75 embryos (revised Fig.2B), which revealed that the number dramatically decreases at E8.75. Upregulation of T would be necessary for inducing expression of PGC specifiers (Aramaki et al., 2013) at least in case of Otx2 is normal (Zhang et al., 2018), but not later on.

6. Figure 2 at E8.75 there are a large number of Venus positive cells that are quite distant to the main cluster, including some that are very anterior. Are these PGCs that have been specified ectopically? Or have they migrated away? Would staining for another marker be useful to identify what these cells are?

As the reviewer pointed out, some Venus positive PGCs, particularly T negative ones, are away from the cluster. Since T negative PGCs are a bit advanced from nascent PGCs as we have shown in the revised Fig.2B, these PGCs might be moving out ready for migration as reported previously in mice using in toto imaging (McDole et al., 2018). We have added in a sentence and the citation in the revised manuscript (P8 lines 15-16).

7. Figure S3B – the correlation between Sox2 and Venus is very strong at E9.5, but there appears to be a number of PGCs with low/absent Oct4 – in particular those cells which have migrated furthest from the allantois. Is this really the case?

We have carefully rechecked the expression of Oct4 in Venus positive rat PGCs, and found they all express Oct4 (revised Fig.S3B). Our single cell RT-qPCR data shows that expression of Oct4 is relatively homogeneous but shows slight heterogeneity, which might affect the protein levels. Regarding the PGCs located away from the allantois, as discussed above, they are possibly migrating PGCs.

8. Prdm14mut/+ mice: No data is shown to verify the mutation in these mice – please include this in supplementary material. Can this mouse be readily maintained as a heterozygous breeding colony?

We have added in details regarding Prdm14mut/+ rats in revised Fig.S1F,G. They can be readily maintained as a heterozygous breeding colony and, so far, have produced more than a hundred rats, with heterozygous rats born at a Mendelian ratio.

9. Page 9, line 13. 're-acquisition of pluripotency'. This term appears to be used as a direct carry-over from descriptions often used in the mouse. However, other than Sox2, which pluripotency genes are actually turned off and re-expressed during rat PGC specification, and which genes are maintained? (Although Oct4, levels do decrease somewhat it is known in mouse that a 50% reduction in Oct4 actually improves self-renewal of mouse ES cells (Karwacki-Neisius et al., 2013) and so this is unlikely to influence the 'pluripotent character' of rat PGCs.) A great benefit of this new and exciting rat data is to really question which aspects are conserved with mice, and so the authors should be cautious before adopting the principles which have become accepted dogma in mice.

To address the reviewers comment, we focused on dynamics of pluripotency genes and found that the naïve pluripotency genes, Esrrb and Klf2 are re-activated in E9.5 rat PGCs but Klf4 and Tfcp2l1 are not, which is in contrast to human PGCs (revised Fig.4D). We also performed IF for Otx2, a repressor of PGC fate in mice (Zhang et al., 2018), and found that its expression is mutually exclusive with the posterior epiblast and Prdm14 H2Bvenus positive PGCs as in mice (data not shown in the manuscript due to lack of space). Although further functional validation is necessary, so far at least from expression levels, the dynamics of pluripotency genes appear well conserved between mouse and rat.

10. Page 10, Line 19. The study by the Surani laboratory also contributed to the findings described here (Grabole et al., 2013)

We have added it in the revised manuscript.

11. Page 14, line 2. The study by the Ying laboratory should be included here (Li et al., 2008)

We have added this in the revised manuscript.

12. The recent studies from the Payer lab (Mallol et al., 2019) and Surani lab (bioRxiv 563072) should be discussed and cited.

We have added them in the revised manuscript and discuss the latter one in the text (P13 line 24-P14 line 2).

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Second decision letter

MS ID#: DEVELOP/2019/183798

MS TITLE: Germline development in rat revealed by visualization and deletion of *Prdm14*

AUTHORS: Toshihiro Kobayashi, Hisato Kobayashi, Teppei Goto, Tomoya Takashima, Mami Oikawa, Hiroki Ikeda, Reiko Terada, Fumika Yoshida, Makoto Sanbo, Hiromitsu Nakauchi, Kazuki Kurimoto, and Masumi Hirabayashi
ARTICLE TYPE: Techniques and Resources Report

I have now received all the referees reports on the above manuscript, and am about to accept the manuscript after small modifications that are suggested by reviewers 2 and 3. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

Please attend to these reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

See previous review

Comments for the author

See previous review. I am happy with the corrections made by the authors

Reviewer 2

Advance summary and potential significance to field & Comments for the author

All my concerns were appropriately addressed, and the revised manuscript has been substantially improved.

There are a few minor comments below.

1. Page 12, lines 8-10: The expression of Ddx4, Dazl and Mael is missed in Fig.S6C.

2. Page 13, line 4: Fig.6B should be Fig.S6B.

Reviewer 3

Advance summary and potential significance to field

The authors have adequately addressed my comments. I think the paper is now ready for publication in Development and makes an important contribution. Well done.

Comments for the author

I have two minor comments that I think should be addressed prior to publication.

1. Page 3; Line 9: ‘Germline development in mammals is widely divergent with conservation of some key networks across species’. This statement is not supported by the reference cited. There is little evidence in the literature to support the idea that mammalian germline development is ‘widely divergent’. In fact, quite the opposite is true – with many fundamentals surprisingly conserved (including the central role of BMP signaling, requirement for Oct4 and expression of other pluripotency genes, expression of conserved germline markers

– Dazl, Vasa, Dnd1, Nanos etc, similarities in migration). In fact, current evidence suggests that basic developmental mechanisms are conserved, but there are some apparent species-specific quirks. In any case, this statement should be adjusted.

Page 10; Line 15: 'Notably, even in the absence of Prdm14, both mouse PGCs at early-to-mid bud stage and mouse PGC-like cells in vitro which is equivalent to migratory PGCs delay but retain the expression of several germ cell markers as well as pluripotency genes at the transcript level' – this sentence is difficult to understand and should be revised.

Second revision

Author response to reviewers' comments

We thank you all again for your time and effort in reviewing our revised manuscript. We have addressed all the minor comments and suggestions by the reviewers. Here is a point-wise response to the comments from reviewer 2 and 3.

Reviewer 2 Advance Summary and Potential Significance to Field:
All my concerns were appropriately addressed, and the revised manuscript has been substantially improved. There are a few minor comments below.

Thank you very much for carefully reading of our manuscript.

1. Page 12, lines 8-10: The expression of Ddx4, Dazl and Mael is missed in Fig.S6C.

We have added the data in revised Fig.S6C.

2. Page 13, line 4: Fig.6B should be Fig.S6B.

We have fixed the number.

Reviewer 3 Advance Summary and Potential Significance to Field:
The authors have adequately addressed my comments. I think the paper is now ready for publication in Development and makes an important contribution. Well done.

Thank you very much for valuable comments and suggestions.

I have two minor comments that I think should be addressed prior to publication.

1. Page 3; Line 9: 'Germline development in mammals is widely divergent with conservation of some key networks across species'. This statement is not supported by the reference cited. There is little evidence in the literature to support the idea that mammalian germline development is 'widely divergent'. In fact, quite the opposite is true – with many fundamentals surprisingly conserved (including the central role of BMP signaling, requirement for Oct4 and expression of other pluripotency genes, expression of conserved germline markers – Dazl, Vasa, Dnd1, Nanos etc, similarities in migration). In fact, current evidence suggests that basic developmental mechanisms are conserved, but there are some apparent species-specific quirks. In any case, this statement should be adjusted.

We agree the reviewer's concern and have removed the statement in the revised manuscript as follows (P3 line 9-10):
Germline development in mammals has been extensively studied in the mouse model system. …

Page 10; Line 15: 'Notably, even in the absence of Prdm14, both mouse PGCs at early-to-mid bud stage and mouse PGC-like cells in vitro which is equivalent to migratory PGCs delay but retain the

expression of several germ cell markers as well as pluripotency genes at the transcript level' – this sentence is difficult to understand and should be revised.

We have simplified the sentence as follows (P10 line 13-15):
…. Notably, even in the absence of Prdm14, mouse PGCs delay but retain the expression of several germ cell markers as well as pluripotency genes at the transcript level (…

Third decision letter

MS ID#: DEVELOP/2019/183798

MS TITLE: Germline development in rat revealed by visualization and deletion of *Prdm14*

AUTHORS: Toshihiro Kobayashi, Hisato Kobayashi, Teppei Goto, Tomoya Takashima, Mami Oikawa, Hiroki Ikeda, Reiko Terada, Fumika Yoshida, Makoto Sanbo, Hiromitsu Nakauchi, Kazuki Kurimoto, and Masumi Hirabayashi
ARTICLE TYPE: Techniques and Resources Report

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