Paternally inherited H3K27me3 affects chromatin accessibility in mouse embryos produced by round spermatid injection

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MS TITLE: Paternally inherited H3K27me3 impacts zygotic genome activation in round spermatid injection

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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. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee’s comments, and we will look over this and provide further guidance.

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

This paper by Mizuki Sakamoto and colleagues reports that mouse preimplantation embryos generated by intracytoplasmic injection of round spermatids (ROSI) have differential gene expression at the one-and 2-cell stages (1CE and 2CE respectively). The main outcomes of the paper are the delineation of differential gene expression in 1CE and 2CE when they were generated by ROSI instead of ICSI, and the finding that male pronuclei in ROSI-generated zygotes contain more trimethylated histone H3 in lysine p27 (H3K27me3). The authors hypothesize that during late spermiogenesis there may be selective removal of H3T marked with bivalent or H3K27 trimethylation marks (H3K27me3), and replacement with H3.3 without the H3K27me3 mark. In mature sperm, there would consequently be less of the inactivating H3K27me3 mark, and when ICSI, but not ROSI, is performed, the chromatin of promoter regions would therefore be more open and active in 1CE, enhancing gene expression. If ROSI is performed, the inactivating H3K27me3 mark would be more prevalent in 1CE, and gene expression weakened, according to this model. The investigation addresses the well-known, but little understood, observation that ROSI is much less effective than ICSI, with very small percentages of concepti reaching full term development compared to ICSI. This is therefore a timely investigation, and significant.
The ATACseq and RNAseq experiments, as well as the analyses of large data sets from other groups appear to be well done and the resulting data are interesting.
The major weakness of this paper is that the main premise for data interpretation appears to be limited to chromatin remodeling steps in spermiogenesis that involve the replacement of H3T by H3.3., which has been proposed to happen mainly in genes actively transcribed during spermiogenesis.
A number of alternative interpretations are possible, but not discussed in the manuscript.
This study is based on sound data, that support the main conclusion that there are abnormal epigenetic chromatin signatures in ROSI embryos inherited from round spermatids as opposed to mature sperm, which that alters gene expression in ROSI embryos. If appropriately revised and data were evaluated in a more unbiased way, in particular regarding the proposed model, this paper may make a significant contribution to the field.
In summary, while data shown appear to be valid and interesting, their interpretation is not well reasoned. However, general conclusions drawn (such as that there are abnormal epigenetic chromatin signatures in ROSI embryos that alter gene expression) are supported, but the proposed mechanistic model is not very compelling.

Comments for the author

Please see positive comments in previous section.
There are some major concerns:
1. The conclusion that “Collectively, these results indicate that abnormal transcriptome in 2-cell ROSI-embryos is characterised by precocious activation of embryonic genes, enhanced degradation of maternal RNA and insufficient activation of 2-cell-specific genes” (Line 170) is very broad and mostly based on observations made by analyses of ATACseq and RNAseq data. However, these observations do not necessarily support the proposed model (Figure 6). As a general concern, data interpretation in this paper seems to be based on a simplistic view of the complex events that take place during spermiogenesis and after fertilization. For example, Figure 4 shows that pronuclei (PN) formed by sperm used for ICSI have less H3K27me3 (Figure 4) than pronuclei from round spermatids after a ROSI procedure. Given that during late spermatid maturation (elongating spermatids, not used for ROSI) approximately 95% of all histones are removed from the spermatid nucleus and replaced by protamines, this result does not provide evidence that H3K27me3 marks are selectively enriched in PN in ROSI zygotes. Because all histones that are removed from the spermatid nucleus are likely to carry histone modifications, including H3K27me3, but also H3K4me3, H4 acetylation in lysine residues 5, 8, 12 and 16, as well as several dozens of other histone modifications with gene activating or silencing functions H3K27me3 is naturally less represented in mature sperm than in round spermatids
- but also all of those other epigenetic histone markings will be less represented in paternal PN of ICSI zygotes. Except for H3K9me3 and H3K4me3 apparently none of these other histone modifications have been investigated.

Therefore, drawing the conclusion that the observed gene expression effects or ATAC-seq results are due to H3K27me3 or H3K4me3 marks in ROSI embryos is not possible based on the limited data presented. In addition, the replacement of protamine from the male PN generated from a mature sperm with unmarked maternal histones stored in the oocyte will have a significant impact on the complement and frequencies of epigenetic marks in PN chromatin, which is disregarded in the proposed model as well, along with DNA demethylation and other reprogramming events that take place at that time.
2. Another conclusion that cannot be drawn based on the presented data is that carryover RNA drove the RNAseq analysis in the ROSI 1CE. Based on the data presented, it cannot be excluded that these genes were actively expressed from the paternal genome complement in the zygote because the round spermatids used for ROSI have probably not undergone global transcriptional silencing in the testis, which happens only during spermatid elongation. Elongated spermatids were not used for ROSI.

Minor concerns:
1. Figure 2E, F needs additional explanation how data sets generated by other groups were used to create the heatmap.
2. Figures 1C and 3D appear to support ambiguous conclusions regarding chromatin accessibility in ICSI and ROSI embryos. Figure 3D suggests that ATACseq signals were generally lower in ROSI embryos. Figure 1C suggests that chromatin remodeling normally occurs in ROSI-embryos (line 106, 107). In Figure 3D, data suggest chromatin accessibility at regions generally marked by active H3K4me3 in early embryos is suppressed in ROSI 1-cell embryos possibly due to inheritance of repressive chromatin status from round spermatids (lines 209-213). While the latter seems reasonable, there should be more explanation and some indication to what extent only bivalently marked genes were differentially expressed in ROSI embryos.
3. The title should clearly reflect that mice were used in this study, not human subjects. Mice are not mentioned until line 290 of the manuscript.

Reviewer 2

Advance summary and potential significance to field

This manuscript seeks to examine why embryos derived from round spermatid injection, compared with intracytoplasmic sperm injection, have a lower efficiency of producing healthy offspring. They produce ATAC-seq and RNA-seq profiles in 1-cell and 2 cell embryos derived from each fertilization method and compare the differential ATAC peaks and differential expressed genes with published chromatin marks during spermiogenesis and early embryonic development. They observe that genes associated with regions that are less accessible in 1-cell embryos derived from round spermatid injection (compared with intracytoplasmic sperm injection) tend to be more lowly expressed. Moreover, these regions are normally marked by H3K27me3 in round spermatids, but not in mature sperm. They observe higher H3K27me3 in the male pronucleus of 1 and 2-cell round spermatid injection embryos, suggesting that skipping the reprogramming of H3K27me3 in spermiogenesis by injecting round spermatids results in some inheritance of paternal H3K27me3 which impacts chromatin accessibility and expression in the embryo. This is an interesting and novel finding that makes sense and one that may be impactful for the field as it considers how the success of round spermatid injection can be improved in the future.

Comments for the author

There are several areas I believe need to be improved in order to support the authors claims including statistical analyses and new comparisons with published data. In addition, the authors need to temper their claims in some areas as the data do not currently support their conclusions, as detailed below.
Most importantly, the authors needs to quantitate and statistically analyse their data to support their claims throughout the manuscript, rather than making qualitative statements about the data on which they rely for their conclusions. At the moment the statistical analysis is limited to defining differentially accessible peaks and differentially expressed genes, but not the comparisons of these groups to other data. The areas needing statistical analyses include:

- The authors claim that cluster 2 genes are upregulated from 1 to 2 cell stage (line 116). The authors need to assess this statistically and provide statistical results. The difference between 1 and 2 cell stage is very minor, and appears equivalent to that in cluster 1.
- Fig 3B considers where ATAC peaks are found and say those that are more accessible in 1 cell ROSI embryos are more common in intronic and intergenic intervals. This requires a statistical comparison.
- Fig S3B presents the chromatin marks at regions that are more open or more closed in ROSI 1-cell embryos compared with ICSI. The authors claim there are differences in H3K4me3 and H3K27me3 at these regions between different timepoints based on published data, but they don't provide a control for other randomly selected regions of the genome. This is required and will enable a more powerful statistical analysis to be made.
- Fig. 3C and D. Again to say there are significant changes or not requires statistical analysis
- Fig. 4C/D no mention of replication is provided in the figure. The number of nuclei scored for each stage needs to be provided and potentially a wide-field picture of more embryos and more sperm/ round spermatids.
- Fig. 5C similarly to S3B above, a statistical analysis is required of the enrichment or otherwise at specific regions of the genome, compared to a random set of permutated ATAC-seq peaks. The authors claim bivalency, but to make this claim they need to show a heatmap or correlation so each region can be checked for both H3K4me3 and H3K27me3 rather than that on average they have both.

More details of the genomic analyses are also required, including:
- Where are the peaks called for the 1-cell ATACseq data in Fig 1C. Can you provide a list of peaks as a supplementary table and mark on the figure panel under the tracks. What is the genomic interval shown in Fig 1C?
- K-means clustering for Fig. 1D – it is not clear which ATAC-seq datasets were used for the clustering. Was it ICSI or ROSI at 1 or 2-cell or both? This needs to be clear in the text and the figure legend.
- The ATAC signal decreasing from 1 to 2 cell for genes that apparently increase in expression seems counterintuitive and deserves further explanation (Fig 1D).
- Fig 1D The authors do not mention H3K9me3 at all, however there is a pattern of some H3K9me3 at the promoters of cluster 1 and cluster 2 genes. This needs some explanation.

There are areas where the data don't support the claim, particularly with regards to disruption of zygotic genome activation mentioned in the title, abstract, results and discussion. Based on the differential gene expression analysis, the authros observe a downregulation of transcripts that include maternal effect genes, downregulation of some zygotic genome activation genes (but not Dux4 itself) and upregulation of embryonic genes. They conclude that they have more rapid maternal RNA degradation, precocious gene activation and disrupted ZGA. However, the same expression profile may be expected for an embryo that is slightly accelerated in development, as the ZGA genes are only on transiently, the maternal RNAs degrade over time, and if ZGA has occurred the embryonic genes will be activated. Can the authors perform PCA or correlation analyses to see if the ROSI 2-cell embryos are developmentally advanced or otherwise provide additional support for ZGA disruption, as opposed to accelerated?

Indeed, the model is built around predominantly the more closed chromatin regions in the ROSI embryos, which tend to be more lowly expressed and H3K27me3 enriched in round spermatids. While the IF data nicely support their model, the model doesn't incorporate the larger number of regions that are more open, and the rest of the differentially expressed genes. For example if there is more embryonic gene expression how does this fit with more repressive marks inherited from the round spermatid? Presumably different regions of the genome, but this needs to be spelled out and discussed in more depth to wrap the paper together. This aspect requires discussion even if it cannot be currently built into the model.

The current manuscript only discusses H3K27me3 retention, however the H3K9me2 are analysed in Fig. 4 by IF and also appear may be retained, but are never mentioned. Comment should be added to the Figure 4 text in the results and the discussion to broaden.

The authors could extend their work in future to include allele-specific chromatin mark ChIP-seq in their embryos along with allele-specific RNA-seq to properly discern the effect on the paternal genome and downstream effects also found on the maternal genome.

Minor comments:
- Differentially expressed genes from early figures compared with Figure 5 can be confusing as they've come about either by a standard DE genes analysis vs considering ATAC-seq peaks. This needs to be made clearer in Figure 5.
- Line 49-50 This sentence is confusing, not clear what is meant by molecular property of ROSI – presumably what goes awry in ROSI?
- Line 72 – need additional refs for H3K27me3 inherited epigenetic mark
- Lin 118-120. Needs further explanation here as it is counterintuitive to have more closed chromatin but apparently activation.
- Line 139 – several DEG – better to give the number
- Line 194 – should say normally active regions as you haven't tested this in the ROSI embryos
- Line 232 States lower chromatin accessibility only mildly affects transcription, but importantly more open chromatin had no effect at all – needs mentioning.

Reviewer 3

Advance summary and potential significance to field

In this manuscript, the authors investigate the mechanism underlying the developmental deficiency of ROSI embryos by analyzing their transcriptome chromatin accessibility, and epigenetic marks. It is shown that in ROSI 1-cell embryos, H3K27me3, which decreases during the transition from round spermatid to mature sperm, remains after fertilization, leading to the formation of low accessible chromatin. The authors also demonstrate that in ROSI 2-cell embryos the expression of the genes with relatively lower accessible promoters (when compared to ICSI embryos) are at a low level and that H3K27me3 level in the promoter regions is much higher. From these results, the authors suggest that in ROSI embryos, H3K27 marks in round spermatids remains after fertilization, which causes the formation of a low accessible chromatin and abnormal gene expression leading to the deficiency in the development.

The results provided in this manuscript are interesting. This is the first report to suggest the molecular mechanism underlying the deficiency in the development of ROSI embryos. In addition, it also provides a clue about the process by which male gametes acquire the competence to complete the development after fertilization.

Comments for the author

Some additional analyses are required to warrant the conclusions in this manuscript (they are described below as major points). In addition, there are some issues which are not critical but should be addressed.
(major points)
The authors conclude that the higher level of H3K27me3 is associated with abnormal gene expression at the 2-cell stage in ROSI embryos (Fig. 5C, line 245-246). Nevertheless, they emphasize the differences of chromatin accessibility and epigenetic marks (including H3K27me3) at the 1-cell stage (line 186-190 line 206-207, Fig.3D) and does not show those differences at the 2-cell stage. It is important to show the results of analyses for the relation among the chromatin accessibility, epigenetic marks (especially, H3K27me3) and gene expression (using RNAseq data) at the 2-cell stage, e.g., as shown for 1-cell stage in Fig. 3D.
Fig. 1D, Line 115-116: There is no point in analyzing the correlation between the results of ATAC-seq and RNA-seq in 1-cell embryos, because most of transcripts in 1-cell embryos are maternal mRNAs which were transcribed and accumulated during the growth of oocytes. In this context, it seems odd that there is correlation between the promoter signals of ATAC-seq and the transcriptome in 1-cell embryos, because it suggests that chromatin remodeling does not occurs during/after fertilization and that the condition of chromatin structure in growing oocytes is still maintained in 1-cell embryos. Some explanation for these results should be required.
Line 191-192 and 194-195 Fig. S3B: The authors claim that chromatin accessibility decreased at active chromatin regions in 1-cell ROSI-embryos.

However, not only ROCI-low but also ROCI-high peaks appear to be enriched in H3K4me3 and depleted in H3K27me3. Furthermore, H3K27me3 appears to be rather higher in ROSI-low peaks than

ROSI-high ones. In addition, chromatin accessible regions are generally localized around the active genes which are enriched in H3K4me3 and depleted in H3K27me3. To warrant their claim, the enrichment of these modifications in ROSI-low peaks should be compared to those of the whole ATAC peaks or “distal” ATAC ones.
(minor points)
Are the development progression rates the same between ROSI and ICSI embryos? If they are different, the differences of the results of RNA-seq and ATAC-seq may be due to the different developmental stages.
Line 153-155, 170: One third of the 2-cell ROSI-high DEG group are expressed at the 2-cell stage (represented as green bar in Fig. 2E). The upregulation of these genes by ROSI are due to not "precocious" but "increased expression".
Fig. 5A: The bars representing the average values seem to be missing in the box blots of distal peaks.
Line 231-232, "while the tendency of downregulation was clearly observed in this gene group, only a few DEGs were included (Fig. 5B),": I do not understand this sentence. Please rewrite it to clearly convey the authors' meaning.
Line 273: Where is “these regions”?
Line 278: The phrase, "the blastocyst stage of ROSI-embryo development", would be changed into "the development of ROSI-embryos to the blastocyst stage".
What stage of development are ROSI-embryos delayed and/or arrested at? It is important to discuss about how the abnormal deposition of H3K27me3 at the 2-cell stage causes the delay/arrest of development at that stage in ROSI-embryos.
Fig. 6: I would like to suggest it to the authors to include the condition of histone modifications in 1-cell embryos and both of the conditions of histone modifications and ATAC-seq peak in 2-cell embryos in the schematic view.

First revision

Author response to reviewers' comments

Point-by-point Responses to the Reviewers:
We appreciate the time and efforts that the reviewers put into reviewing our manuscript and find all helpful comments to improve our manuscript. According to their suggestions, we have performed additional experiments and analyses, the outcomes of which further support our conclusions. In this revised manuscript, we removed some of the RNA-seq results presented in our previous manuscript and mainly focused on changes in chromatin accessibility. Accordingly, we changed the title to “Paternally inherited H3K27me3 affects chromatin accessibility in mouse embryos produced by round spermatid injection”, which we think well represents the main message of this paper. By incorporating the helpful comments from the reviewers, we have also revised the main text and figures, which we believe has improved the clarity and readability of our manuscript.

Reviewer 1

Reviewer 1 Advance summary and potential significance to field

This paper by Mizuki Sakamoto and colleagues reports that mouse preimplantation embryos generated by intracytoplasmic injection of round spermatids (ROSI) have differential gene expression at the one-and 2-cell stages (1CE and 2CE, respectively). The main outcomes of the paper are the delineation of differential gene expression in 1CE and 2CE when they were generated by ROSI instead of ICSI, and the finding that male pronuclei in ROSI-generated zygotes contain more trimethylated histone H3 in lysine p27 (H3K27me3). The authors hypothesize that during late spermiogenesis there may be selective removal of H3T marked with bivalent or H3K27 trimethylation marks (H3K27me3), and replacement with H3.3 without the H3K27me3 mark. In mature sperm, there would consequently be less of the inactivating H3K27me3 mark, and when ICSI, but not ROSI, is performed, the chromatin of promoter regions would therefore be more open and active in 1CE, enhancing gene expression. If ROSI is performed, the inactivating H3K27me3 mark would be more prevalent in 1CE, and gene expression weakened, according to this model.

The investigation addresses the well-known, but little understood, observation that ROSI is much less effective than ICSI, with very small percentages of concepti reaching full term development compared to ICSI. This is therefore a timely investigation, and significant. The ATACseq and RNAseq experiments, as well as the analyses of large data sets from other groups appear to be well done and the resulting data are interesting. The major weakness of this paper is that the main premise for data interpretation appears to be limited to chromatin remodeling steps in spermiogenesis that involve the replacement of H3T by H3.3., which has been proposed to happen mainly in genes actively transcribed during spermiogenesis. A number of alternative interpretations are possible, but not discussed in the manuscript.
This study is based on sound data, that support the main conclusion that there are abnormal epigenetic chromatin signatures in ROSI embryos inherited from round spermatids as opposed to mature sperm, which that alters gene expression in ROSI embryos. If appropriately revised and data were evaluated in a more unbiased way, in particular regarding the proposed model, this paper may make a significant contribution to the field.
In summary, while data shown appear to be valid and interesting, their interpretation is not well reasoned. However, general conclusions drawn (such as that there are abnormal epigenetic chromatin signatures in ROSI embryos that alter gene expression) are supported, but the proposed mechanistic model is not very compelling.

Reviewer 1 Comments for the author
Please see positive comments in previous section.
There are some major concerns:

1. The conclusion that “Collectively, these results indicate that abnormal transcriptome in 2-cell ROSI-embryos is characterised by precocious activation of embryonic genes, enhanced degradation of maternal RNA and insufficient activation of 2-cell-specific genes” (Line 170) is very broad and mostly based on observations made by analyses of ATACseq and RNAseq data. However, these observations do not necessarily support the proposed model (Figure 6). As a general concern, data interpretation in this paper seems to be based on a simplistic view of the complex events that take place during spermiogenesis and after fertilization. For example, Figure 4 shows that pronuclei (PN) formed by sperm used for ICSI have less H3K27me3 (Figure 4) than pronuclei from round spermatids after a ROSI procedure. Given that during late spermatid maturation (elongating spermatids, not used for ROSI) approximately 95% of all histones are removed from the spermatid nucleus and replaced by protamines, this result does not provide evidence that H3K27me3 marks are selectively enriched in PN in ROSI zygotes. Because all histones that are removed from the spermatid nucleus are likely to carry histone modifications, including H3K27me3, but also H3K4me3, H4 acetylation in lysine residues 5, 8, 12 and 16, as well as several dozens of other histone modifications with gene activating or silencing functions, H3K27me3 is naturally less represented in mature sperm than in round spermatids- but also all of those other epigenetic histone markings will be less represented in paternal PN of ICSI zygotes. Except for H3K9me3 and H3K4me3, apparently none of these other histone modifications have been investigated. Therefore, drawing the conclusion that the observed gene expression effects or ATAC-seq results are due to H3K27me3 or H3K4me3 marks in ROSI embryos is not possible based on the limited data presented. In addition, the replacement of protamine from the male PN generated from a mature sperm with unmarked maternal histones stored in the oocyte will have a significant impact on the complement and frequencies of epigenetic marks in PN chromatin, which is disregarded in the proposed model as well, along with DNA demethylation and other reprogramming events that take place at that time.

Response:
We would like to thank these valuable comments. We agree that there should be many paternally inherited epigenetic factors which may affect embryonic gene expression in ROSI. In this study, we mainly focused on our ATAC-seq and RNA-seq data generated for ICSI and ROSI embryos, and searched for epigenetic signatures that can explain altered chromatin accessibility or gene expression. We then found that the genomic regions showing lower chromatin accessibility in ROSI embryos correspond to the H3K27me3-marked regions in round spermatids. In addition to H3K27me3, we additionally analyzed H3K4me3, H3K4me1, and H3K36me3 in round spermatids and sperm using publicly available datasets, but H3K27me3 only was the histone mark that could explain the altered chromatin accessibility (new Fig. S4C). However, we never exclude the possibility that other histone marks or RNA or protein transmitted from round spermatids might be involved in altered chromatin accessibility in ROSI-embryos. To make these points clearer, we

investigated the potential involvement of transcription factors that can be transmitted from round spermatids (Fig. S7A). Although, unfortunately, the results from this analysis was not conclusive, we believe that the requirement of further analysis from different aspects is now emphasized by this data. As we understand that we do not provide direct evidence that H3K27me3 in round spermatids lowers chromatin accessibility and gene expression in ROSI embryos, we carefully revised our manuscripts (e.g., Line 312-327 in Discussion).

2. Another conclusion that cannot be drawn based on the presented data is that carryover RNA drove the RNAseq analysis in the ROSI 1CE. Based on the data presented, it cannot be excluded that these genes were actively expressed from the paternal genome complement in the zygote because the round spermatids used for ROSI have probably not undergone global transcriptional silencing in the testis, which happens only during spermatid elongation. Elongated spermatids were not used for ROSI.

Response:
In order to further examine whether ROSI embryos have RNA carried over from round spermatids, we performed qRT-PCR using DRB, a potent inhibitor of RNA pol II, to block de novo transcription in ROSI-embryos. Importantly, expression of Tp1, Tp2, and Smcp, identified as upregulated genes in ROSI-embryos, was consistently higher even after the DRB treatment in ROSI-embryos than that in ICSI-embryos (new Fig. 2E). Thus, we now provide evidence that the presence of RNA carried over from round spermatids is one of the causes of differential gene expression in ROSI-embryos.

Minor concerns:

1. Figure 2E, F needs additional explanation how data sets generated by other groups were used to create the heatmap.

Response:
Based on comments from another reviewer (reviewer 2), we considered that the results previously shown as Fig. 2E and F are not meaningful in our paper and rather weaken our main message. Therefore, we removed them in our revised manuscript.

2. Figures 1C and 3D appear to support ambiguous conclusions regarding chromatin accessibility in ICSI and ROSI embryos. Figure 3D suggests that ATACseq signals were generally lower in ROSI embryos. Figure 1C suggests that chromatin remodeling normally occurs in ROlSI-embryos (line 106, 107). In Figure 3D, data suggest chromatin accessibility at regions generally marked by active H3K4me3 in early embryos is suppressed in ROSI 1-cell embryos possibly due to inheritance of repressive chromatin status from round spermatids (lines 209-213). While the latter seems reasonable, there should be more explanation and some indication to what extent only bivalently marked genes were differentially expressed in ROSI embryos.

Response:
As shown in Fig. 3A, differential accessible chromatin regions between ROSI- and ICSI-embryos occupied only less than 10% of total ATAC peaks. Therefore, most of the accessible regions are unaffected in ROSI embryos as seen in Fig. 1C. However, when focused on the regions showing lower chromatin accessibility, these regions showed H3K27me3 and moderate H3K4me3 enrichment in round spermatids (Fig. 3C and new Fig. S4C,D). We analyzed expression of the genes associated with these altered chromatin accessibility and observed weaker expression in 2-cell embryos (Fig. 5A). Furthermore, extracting the genes associated with both H3K27me3 and H3K4me3 enrichment at their promoter regions in round spermatid revealed that these bivalently marked genes mainly contribute to the weaker gene expression in ROSI-embryos (Fig. S6A, and see Fig. 3C). We added a clear explanation for these results in the revised manuscript (line 245-247).

3. The title should clearly reflect that mice were used in this study, not human subjects. Mice are not mentined until line 290 of the manuscript.

Response:
We apologize that this point was not clear. Accordingly, the title was changed to “Paternally inherited H3K27me3 affects chromatin accessibility in mouse embryos produced by round spermatid injection”, and we stated that mouse embryos were used in this study (Line 80 –81).

Reviewer 2
Advance summary and potential significance to field
This manuscript seeks to examine why embryos derived from round spermatid injection, compared with intracytoplasmic sperm injection, have a lower efficiency of producing healthy offspring. They produce ATAC-seq and RNA-seq profiles in 1-cell and 2 cell embryos derived from each fertilization method and compare the differential ATAC peaks and differential expressed genes with published chromatin marks during spermiogenesis and early embryonic development. They observe that genes associated with regions that are less accessible in 1-cell embryos derived from round spermatid injection (compared with intracytoplasmic sperm injection) tend to be more lowly expressed. Moreover, these regions are normally marked by H3K27me3 in round spermatids, but not in mature sperm. They observe higher H3K27me3 in the male pronucleus of 1 and 2-cell round spermatid injection embryos, suggesting that skipping the reprogramming of H3K27me3 in spermiogenesis by injecting round spermatids results in some inheritance of paternal H3K27me3 which impacts chromatin accessibility and expression in the embryo. This is an interesting and novel finding that makes sense and one that may be impactful for the field as it considers how the success of round spermatid injection can be improved in the future.

Reviewer 2 Comments for the author
There are several areas I believe need to be improved in order to support the authors claims including statistical analyses and new comparisons with published data. In addition, the authors need to temper their claims in some areas as the data do not currently support their conclusions, as detailed below.

Most importantly, the authors needs to quantitate and statistically analyse their data to support their claims throughout the manuscript, rather than making qualitative statements about the data on which they rely for their conclusions. At the moment the statistical analysis is limited to defining differentially accessible peaks and differentially expressed genes, but not the comparisons of these groups to other data. The areas needing statistical analyses include:

Response:
We apologize that data quantification was not sufficiently performed. We now add quantified data throughout the manuscript, which can be also seen in our response to the comments from this reviewer.

-The authors claim that cluster 2 genes are upregulated from 1 to 2 cell stage (line 116). The authors need to assess this statistically and provide statistical results. The difference between 1 and 2 cell stage is very minor, and appears equivalent to that in cluster 1.

Response:
We would like to thank these important comments. We now show gene expression levels in each cluster by box plots and apply a statistical analysis (new Fig. S1F).

-Fig 3B considers where ATAC peaks are found and say those that are more accessible in 1 cell ROSI embryos are more common in intronic and intergenic intervals. This requires a statistical comparison.

Response:
We reanalyzed the data by integrating randomly selected peaks for comparison. The results showed significant enrichment of differential ATAC peaks within the intronic or intergenic regions (Fisher's exact test, new Fig. S3A)

-Fig S3B presents the chromatin marks at regions that are more open or more closed in ROSI 1-cell embryos compared with ICSI. The authors claim there are differences in H3K4me3 and H3K27me3 at these regions between different timepoints based on published data, but they don't provide a control for other randomly selected regions of the genome. This is required and will enable a more powerful statistical analysis to be made.

Response
We would like to thank this reviewer for these important comments. We analyzed the enrichment of H3K4me3 and H3K27me3 at the randomly selected ATAC peak regions as well (Fig. S3C) and

quantified the enrichment of H3K4me3 (Fig. S3D). This shows that the 1-cell ROSI-high peak regions are characterized by weak enrichment of H3K4me3 compared to those at the randomly selected ATAC peak regions, while the statistical analysis also indicated that the 1-cell ROSI-low peak regions have slightly weaker enrichment of H3K4me3 compared to those at the randomly selected ATAC peak regions. The text was accordingly revised (Line 181– 189).

-Fig. 3C and D. Again to say there are significant changes or not requires statistical analysis

Response:
We quantified the ATAC-seq data and performed statistical analysis (new Fig. S4A, B). The statistical difference was tested by Wilcoxon rank-sum test. The text was revised upon these additional analyses (Line 193-205).

-Fig. 4C/D no mention of replication is provided in the figure. The number of nuclei scored for each stage needs to be provided and potentially a wide-field picture of more embryos and more sperm/ round spermatids.

Response:
According to this suggestion, we replaced the images with wider view images. Sample numbers were added to the figure legend.

Fig. 5C similarly to S3B above, a statistical analysis is required of the enrichment or otherwise at specific regions of the genome, compared to a random set of permutated ATAC-seq peaks. The authors claim bivalency, but to make this claim they need to show a heatmap or correlation so each region can be checked for both H3K4me3 and H3K27me3 rather than that on average they have both.

Response:
We would like to thank this insightful comment. We included randomly selected promoter regions for the analysis. In addition to metagene plots, we newly provide heatmaps and boxplots that are associated with statistical analyses (new Fig. 5C, D and Fig. S6B).

More details of the genomic analyses are also required, including:
-Where are the peaks called for the 1-cell ATACseq data in Fig 1C. Can you provide a list of peaks as a supplementary table and mark on the figure panel under the tracks. What is the genomic interval shown in Fig 1C?

Response:
As requested, we provide an excel file of the list of peaks as Table S1. We also show the peak regions and genomic intervals in Fig. 1C.

-K-means clustering for Fig. 1D – it is not clear which ATAC-seq datasets were used for the clustering. Was it ICSI or ROSI at 1 or 2-cell or both? This needs to be clear in the text and the figure legend.

Response:
We modified the main text as shown below:

Revised text, (Line 116-):
For a more detailed analysis of open chromatin regions, we classified gene promoter regions into four clusters by k-means clustering based on the strength of ATAC peaks of 1-cell ROSI-, 1-cell ICSI-, 2-cell ROSI- and 2-cell ICSI-embryos.

-The ATAC signal decreasing from 1 to 2 cell for genes that apparently increase in expression seems counterintuitive and deserves further explanation (Fig 1D).

Response:
We would like to thank this important suggestion. We agree that more explanation was necessary, so we added some explanation as follows:

Revised text, (Line 122-)
Notably, in clusters 1, 2, and 3, we observed gene expression upregulation during development to the 2-cell stage (Fig. S1F), although ATAC signals at their promoters diminished (Fig. 1D). These findings suggest that promoter accessibility correlates with transcriptional output, however, is not the determinant. Therefore, other factors than promoter accessibility may cooperatively fine tune transcriptional output during ZGA.

-Fig 1D The authors do not mention H3K9me3 at all, however there is a pattern of some H3K9me3 at the promoters of cluster 1 and cluster 2 genes. This needs some explanation.

Response:
We apologize that we did not explain this point. We modified the corresponding section as follows:

Revised text, (Line 111-)
H3K9me3 was enriched at the transcription start sites (TSSs) associated with relatively high accessibility at the 2-cell stage. This may reflect a non-repressive function of H3K9me3 at these embryonic stage (Burton et al., 2020; Wang et al., 2018).

There are areas where the data don't support the claim, particularly with regards to the disruption of zygotic genome activation mentioned in the title, abstract, results and discussion. Based on the differential gene expression analysis, the authors observe a downregulation of transcripts that include maternal effect genes, downregulation of some zygotic genome activation genes (but not Dux4 itself) and upregulation of embryonic genes. They conclude that they have more rapid maternal RNA degradation, precocious gene activation and disrupted ZGA. However, the same expression profile may be expected for an embryo that is slightly accelerated in development, as the ZGA genes are only on transiently, the maternal RNAs degrade over time, and if ZGA has occurred the embryonic genes will be activated. Can the authors perform PCA or correlation analyses to see if the ROSI 2-cell embryos are developmentally advanced or otherwise provide additional support for ZGA disruption, as opposed to accelerated?

Response:
We would like to deeply thank this critical comment. We agree that the alteration of transcriptome of ROSI-embryos, especially at the 2-cell stage, could be a consequence of slight acceleration of developmental clock, and we felt that the descriptive RNA-seq results in this part is not important in our paper and are out of our focus. Furthermore, we also considered that it is difficult to precisely analyze how much ZGA initiation or maternal RNA degradation timing is altered in ROSI-embryos in this revision. Based on these reasons, we decided to remove this part to avoid any confusion and to make our message clearer in the revised manuscript.

Indeed, the model is built around predominantly the more closed chromatin regions in the ROSI embryos, which tend to be more lowly expressed and H3K27me3 enriched in round spermatids. While the IF data nicely support their model, the model doesn't incorporate the larger number of regions that are more open, and the rest of the differentially expressed genes. For example, if there is more embryonic gene expression how does this fit with more repressive marks inherited from the round spermatid? Presumably different regions of the genome, but this needs to be spelled out and discussed in more depth to wrap the paper together. This aspect requires discussion even if it cannot be currently built into the model.

Response:
We would like to thank this important suggestion. We agree that our model only focused on the genomic regions where inherited H3K27me3 from round spermatids affects chromatin accessibility and transcription. However, as this reviewer pointed out, we observed more open chromatin regions as well as upregulated genes in ROSI-embryos. Therefore, in the revised manuscript, we analysed the enrichment of histone marks other than H3K27me3 (H3K4me1, H3K4me3, and H3K36me3) in round spermatids and sperm, but we could not find any clear positive or negative correlation. These analyses further clarified the importance of H3K27me3.
In addition, we tested an alternative possibility that transcriptional factors transmitted from round spermatids might be involved in the acquisition of more open chromatin in ROSI-embryos, however, the result from this analysis was also not conclusive (Fig. 5; also see our response to the major comment #1 from Reviewer 1). Nevertheless, we think that, with these additional data, it is now

emphasized in the revised manuscript that there are many possibilities which potentially affect chromatin status or gene expression in ROSI-embryos. To demonstrate these additional data, we modified the section: (Line 230-235 and 265-286).

The current manuscript only discusses H3K27me3 retention, however the H3K9me2 are analysed in Fig. 4 by IF and also appear may be retained, but are never mentioned. Comment should be added to the Figure 4 text in the results and the discussion to broaden.

Response:
We added some explanations as follows:

Revised text, (Line 220-)
To distinguish paternal and maternal chromatin, we also performed immunostaining for H3K9me2, whose stronger signals were observed on the maternal chromatin at the 1-cell and 2-cell stages (Burton and Torres-Padilla, 2010). Similarly to the previous observation for H3K9me3 (Kishigami et al., 2006), ectopic H3K9me2 signals were observed at the pericentromeric heterochromatin region in male PN of ROSI 1-cell embryos (Fig. 4A, B). On the contrary, at the 1-cell stage, H3K27me3 intensity in male pronuclei in ROSI-embryos was globally higher than that in ICSI-embryos (Fig. 4A, B).

The authors could extend their work in future to include allele-specific chromatin mark ChIP-seq in their embryos along with allele-specific RNA-seq to properly discern the effect on the paternal genome and downstream effects also found on the maternal genome.

Response:
We would like to thank this important suggestion. As the reviewer suggested, we consider that allele-specific histone modification and gene expression analyses are definitely required in our future study. We described this point in Discussion as follows:

Revised text, (Line 323-)
Therefore, identifying the genomic distribution of H3K27me3 in ROSI-derived embryos in an allele-discriminative manner would be crucial given the recent progress in low-input epigenetic analytical techniques. These analyses will clearly pinpoint comprehensive knowledge on not only the causes of low birth rates but also the epigenetic events during spermiogenesis essential for embryonic development.

Minor comments:
-Differentially expressed genes from early figures compared with Figure 5 can be confusing as they've come about either by a standard DE genes analysis vs considering ATAC-seq peaks. This needs to be made clearer in Figure 5.

Response:
To make this point clearer, we modified the corresponding figure legend as follows:

Revised text, (Line 801-)
ATAC peak regions were divided into two groups "distal" and "promoter" peaks. Then, the expression was analyzed on the genes associated with the indicated ATAC peak regions defined as "common", "high", or "low" accessibility at distal or promoter peaks.

-Line 49-50 This sentence is confusing, not clear what is meant by molecular property of ROSI – presumably what goes awry in ROSI?

Response:
We changed the sentence as follows:

Revised text, (Line 48-)
"Therefore, toward its clinical application, it is important to understand at the molecular level why ROSI-embryos show poor development"

-Line 72 – need additional refs for H3K27me3 inherited epigenetic mark

(Line 73-74)
Response: Additional references (Erkek et al., 2013; Lesch et al., 2013; Lesch et al., 2016; Maezawa et al., 2018b; Sin et al., 2015; Teperek et al., 2016) are now cited.

-Lin 118-120. Needs further explanation here as it is counterintuitive to have more closed chromatin but apparently activation.

Response:
We added more explanation/discussion as follows:
(Line 122-126)
Notably, in clusters 1, 2, and 3, we observed gene expression upregulation during development to the 2-cell stage (Fig. S1F), although ATAC signals at their promoters diminished (Fig. 1D). These findings suggest that promoter accessibility correlates with transcriptional output, however, is not the determinant. Therefore, other factors than promoter accessibility may cooperatively fine tune transcriptional output during ZGA.

-Line 139 – several DEG – better to give the number

Response: Line 149-150
We now show the number of DEGs there.

-Line 194 – should say normally active regions as you haven't tested this in the ROSI embryos

Response:
We revised the text, (Line 191).

-Line 232 States lower chromatin accessibility only mildly affects transcription, but importantly more open chromatin had no effect at all – needs mentioning.

Response:
Thank you for your suggestion. We added a sentence as follows:

Revised text, (Line 241),
We observed no changes in gene expression when focused on distal ATAC peaks or ROSI-high ATAC peaks (Fig. 5A).

Reviewer 3
Reviewer 3 Advance summary and potential significance to field
In this manuscript, the authors investigate the mechanism underlying the developmental deficiency of ROSI embryos by analyzing their transcriptome, chromatin accessibility, and epigenetic marks. It is shown that in ROSI 1-cell embryos, H3K27me3, which decreases during the transition from round spermatid to mature sperm, remains after fertilization, leading to the formation of low accessible chromatin. The authors also demonstrate that in ROSI 2-cell embryos, the expression of the genes with relatively lower accessible promoters (when compared to ICSI embryos) are at a low level and that H3K27me3 level in the promoter regions is much higher. From these results, the authors suggest that in ROSI embryos, H3K27 marks in round spermatids remains after fertilization, which causes the formation of a low accessible chromatin and abnormal gene expression, leading to the deficiency in the development.

Major points

The authors conclude that the higher level of H3K27me3 is associated with abnormal gene expression at the 2-cell stage in ROSI embryos (Fig. 5C, line 245-246). Nevertheless, they emphasize the differences of chromatin accessibility and epigenetic marks (including H3K27me3) at the 1-cell stage (line 186-190, line 206-207, Fig.3D) and does not show those differences at the 2-cell stage. It is important to show the results of analyses for the relation among the chromatin accessibility, epigenetic marks (especially, H3K27me3) and gene expression (using RNAseq data) at the 2-cell stage, e.g., as shown for 1-cell stage in Fig. 3D.

Response:
We apologize that these points were not clearly stated in the original manuscript. “1-cell ROSI-low ATAC peak regions”, marked by H3K27me3 in round spermatids, showed lower chromatin accessibility at the 1-cell stage but not at the 2-cell stage. These are represented by Fig. 3C and Fig. S3B. To make this point clearer, we added the following sentences in the revised manuscript:

Revised text, (Line 209):
Notably, this lower chromatin accessibility was cancelled at the 2-cell stage (Fig. 3C and Fig. S3B), thus these changes in accessibility appear to be transient.

Fig. 1D, Line 115-116: There is no point in analyzing the correlation between the results of ATAC-seq and RNA-seq in 1-cell embryos, because most of transcripts in 1-cell embryos are maternal mRNAs which were transcribed and accumulated during the growth of oocytes. In this context, it seems odd that there is correlation between the promoter signals of ATAC-seq and the transcriptome in 1-cell embryos, because it suggests that chromatin remodeling does not occurs during/after fertilization and that the condition of chromatin structure in growing oocytes is still maintained in 1-cell embryos. Some explanation for these results should be required.

Response:
As the reviewer mentioned, 1-cell stage embryos have limited transcriptional activity, and thus most of their transcriptome represent maternal RNA pool as shown in Fig. 1D. We added some explanation on this (Line 119-122). Thus, although ATAC-seq provides snapshots of genome-wide chromatin accessibility, RNA-seq for 1-cell embryos does not necessarily provide the information of zygotic transcription. However, as we think that there is technical difficulty to faithfully detect zygotic transcripts in 1-cell embryos, we used conventional RNA-seq approaches only. As we agree the points raised by this reviewer, we added the following sentences in Discussion:

Revised text, line 297
However, we cannot exclude a possibility that there may be more defects in transcription in 1-cell ROSI-embryos than detected in this study as it is hard to see relatively low amount of zygotic transcripts with a conventional RNA-seq at this stage.

Line 191-192 and 194-195 Fig. S3B: The authors claim that chromatin accessibility decreased at active chromatin regions in 1-cell ROSI-embryos.

However, not only ROCI-low but also ROCI-high peaks appear to be enriched in H3K4me3 and depleted in H3K27me3. Furthermore, H3K27me3 appears to be rather higher in ROSI-low peaks than ROSI-high ones. In addition, chromatin accessible regions are generally localized around the active genes which are enriched in H3K4me3 and depleted in H3K27me3. To warrant their claim, the enrichment of these modifications in ROSI-low peaks should be compared to those of the whole ATAC peaks or “distal” ATAC ones.

Response:
In our additional analyses, we randomly selected accessible ATAC peak regions and used them as a control. We did not use all ATAC peaks as a control because we considered that the number of ATAC peaks analyzed should be similar between the groups compared. We then analyzed the enrichment of the histone marks at these regions. In brief, H3K4me3 was weakly enriched at 1-cell ROSI-high ATAC peak regions while H3K4me3 and H3K27me3 were similarly enriched at the 1-cell ROSI-low ATAC peak regions as in the control regions (Fig. S3C).

Are the development progression rates the same between ROSI and ICSI embryos? If they are different, the differences of the results of RNA-seq and ATAC-seq may be due to the different developmental stages.

Response:
We would like to deeply thank this critical comment. We agree that the alteration of transcriptome in ROSI-embryos, especially those at the 2-cell stage, could be a consequence of slight acceleration of developmental clock, and we felt that descriptive RNA-seq results in this part is not important in our paper as they are out of our focus. Furthermore, we also considered that it is difficult to precisely analyze how much ZGA initiation or maternal RNA degradation timing is altered in ROSI-

embryos. Based on these reasons, we removed this part to avoid any confusion and to make our message clearer in the revised manuscript.

Line 153-155, 170: One third of the 2-cell ROSI-high DEG group are expressed at the 2-cell stage (represented as green bar in Fig. 2E). The upregulation of these genes by ROSI are due to not "precocious" but "increased expression".

Response:
We would like to thank this comment, however, we removed the corresponding results from the revised version as described above. We would like to leave this point for our future study.

Fig. 5A: The bars representing the average values seem to be missing in the box blots of distal peaks.

Response:
We showed the average values in the revised version of Fig. 5A.

Line 231-232, "while the tendency of downregulation was clearly observed in this gene group, only a few DEGs were included (Fig. 5B),": I do not understand this sentence. Please rewrite it to clearly convey the authors' meaning.

Response:
We modified the sentence (Line 248-250).

Line 273: Where is “these regions”?

Response:
We changed the corresponding sentence (Line 312).

Line 278: The phrase, "the blastocyst stage of ROSI-embryo development", would be changed into "the development of ROSI-embryos to the blastocyst stage"

Response:
Thank you for your kind indication. We corrected the sentence (Line 316).

What stage of development are ROSI-embryos delayed and/or arrested at? It is important to discuss about how the abnormal deposition of H3K27me3 at the 2-cell stage causes the delay/arrest of development at that stage in ROSI-embryos.

Response:
As shown in our previous study, preimplantation development to the blastocyst stage was slightly lower in ROSI-embryos (Hirose et al. 2020 Reproduction). Regarding how abnormal presence of H3K27me3 affects ROSI-embryo development, we would like to leave this important question in our future study as this needs lots of additional experiments.

Fig. 6: I would like to suggest it to the authors to include the condition of histone modifications in 1-cell embryos and both of the conditions of histone modifications and ATAC-seq peak in 2-cell embryos in the schematic view.

Response:
Thank you for your advice. We modified the illustration of the proposed model (Fig. 6).

Second decision letter

MS ID#: DEVELOP/2022/200696

MS TITLE: Paternally inherited H3K27me3 affects chromatin accessibility in mouse embryos produced by round spermatid injection

AUTHORS: Mizuki Sakamoto, Daiyu Ito, Rei Inoue, Sayaka Wakayama, Yasuyuki Kikuchi, Li Yang, Erika Hayashi, Rina Emura, Hirosuke Shiura, Takashi Kohda, Satoshi H Namekawa, Takashi Ishiuchi, Teruhiko Wakayama, and Masatoshi Ooga

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 reviewer 3 still expressed some concerns and recommend a further revision of your manuscript before we can consider publication. I do not think experimental challenges are required. Please give a bit more mechanistic interpretations as suggested by this reviewer. 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. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee’s comments, and we will look over this and provide further guidance.

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

Intracytoplasmic sperm injection (ICSI) is a widely used technique in assisted reproduction, where sperm (really late step elongated spermatids)are surgically retrieved from the testis and injected into an oocyte to generate an embryo.
This paper addresses the well-known but poorly understood observation that is is much more difficult to generate such embryos when less highly developed round spermatids are injected. During late spermiogenesis, spermatids undergo a dramatic chromatin remodeling process, where the vast majority of histones are replaced by protamines. In gene promoter regions, this process includes a selective replacement of H3T marked with H3K27 (H3K27me3) or bivalent (H3K4me3/H3K27me3) trimethylation marks with the noncanonical histone variant H3.3 without the H3K27me3 mark, and the authors show that this leads to a net loss of H3K27me3 in mature sperm. The investigators show that there is consequently less of the inactivating H3K27me3 mark, and when ICSI, but not ROSI, is performed, the chromatin of promoter regions would therefore be more open and active in one-and two-cell embryos (2CE), enhancing gene expression. If ROSI is performed, the inactivating H3K27me3 mark would be more prevalent in 1CE and 2CE, and gene expression altered, according to this model. The work therefore represents a timely and significant contribution to the field.
Comments for the author

The authors have responded appropriately to previous concerns and added crucial experiments and important new data to the study. I have no further concerns to level against this manuscript.

Reviewer 2

Advance summary and potential significance to field

This manuscript considers why ROSI embryos have much lower success rates than ICSI embryos. They identify inheritance of H3K27me3 marked chromatin from the round spermatids as being a likely culprit, and suggest there may be some other factors that influence active chromatin, that they haven't yet been able to identify. The data on H3K27me3 is a significant finding, while their additional data opens the door for these authors or others to expand on this work and find additional factors that may be carried over from the round spermatid. As this has direct relevance for the assisted reproduction field, it is of likely to be of high significance for the field.

Comments for the author

The authors have addressed all of my prior concerns. Their revised manuscript incorporates stronger statistical analyses to support their claims and has a more focused narrative that makes the overall story easier to follow. I appreciate their work that attempted to understand the differences in the active regions via studying other chromatin marks and transcription factors. I do not think any further revisions are required.

Reviewer 3

Advance summary and potential significance to field

These issues have been described at the initial review.

Comments for the author

In the revised manuscript, the authors have addressed satisfactorily some of the issues raised in the initial review. However, some serious problems still remain as described below.
The purpose of this study is to elucidate the mechanism causing a low birth rate in ROSI-embryos. However, this manuscript fails in providing a plausible mechanism for it. Although the authors provide the results of analyzing various factors involved in the developmental process, e.g. gene expression, epigenetic modifications and open chromatin sites, they fail in elucidating the relationships among these factors to construct a plausible mechanism. Although the gene expression pattern is different between ROSI- and ICSI-embryos, which seems to be a cause of a low birth rate in ROSI-embryos, the mechanism underlying this difference is not elucidated. In the hypothesis shown in Fig. 6 the genes with H3K27me3 in round spermatids keep this modification at the 1- and 2-cell stages after ROSI. In these embryos, H3K27me3 causes the lower accessibility of chromatin, leading to the suppression of gene expression.
However, only a small part of ROSI-DEGs is identical between 1- and 2-cell (Fig. S2C). Furthermore, most of ROSI-high and low peaks in ATAC analysis are different between 1- and 2-cell (Fig. S3B). These results indicate that the chromatin accessible state and increased expression of a gene is not kept from 1-cell to 2-cell, which is inconsistent with the hypothesis in Fig. 6. Finally Fig. 2E shows that the mRNAs derived from round spermatids are introduced into ROSI-1 cell embryos. In this case, it seems to make no sense to analyze open chromatin and epigenetic modifications in ROSI-1-cell embryos to elucidate the causes of ROSI-high DEGs. Therefore, the manuscript should be thoroughly revised.
In Fig. 3SD, the quantitative analysis should be conducted for H3K27me3 as well as H3K4me3 (H3K27me3 is rather more important).

Second revision

Author response to reviewers' comments

Point-by-point Responses to the Reviewers:

Reviewer 1 Advance summary and potential significance to field

Intracytoplasmic sperm injection (ICSI) is a widely used technique in assisted reproduction, where sperm (really late step elongated spermatids)are surgically retrieved from the testis and injected into an oocyte to generate an embryo. This paper addresses the well-known but poorly understood observation that is is much more difficult to generate such embryos when less highly developed round spermatids are injected. During late spermiogenesis, spermatids undergo a dramatic chromatin remodeling process, where the vast majority of histones are replaced by protamines. In gene promoter regions, this process includes a selective replacement of H3T marked with H3K27 (H3K27me3) or bivalent (H3K4me3/H3K27me3) trimethylation marks with the noncanonical histone variant H3.3 without the H3K27me3 mark, and the authors show that this leads to a net loss of H3K27me3 in mature sperm. The investigators show that there is consequently less of the inactivating H3K27me3 mark, and when ICSI, but not ROSI, is performed, the chromatin of promoter regions would therefore be more open and active in one-and two-cell embryos (2CE), enhancing gene expression. If ROSI is performed, the inactivating H3K27me3 mark would be more prevalent in 1CE and 2CE, and gene expression altered, according to this model. The work therefore represents a timely and significant contribution to the field.

Reviewer 1 Comments for the author
The authors have responded appropriately to previous concerns and added crucial experiments and important new data to the study. I have no further concerns to level against this manuscript.

Response: We would like to thank all the constructive comments from this reviewer which were helpful to improve our manuscript.

Reviewer 2 Advance summary and potential significance to field
This manuscript considers why ROSI embryos have much lower success rates than ICSI embryos. They identify inheritance of H3K27me3 marked chromatin from the round spermatids as being a likely culprit, and suggest there may be some other factors that influence active chromatin, that they haven't yet been able to identify. The data on H3K27me3 is a significant finding, while their additional data opens the door for these authors or others to expand on this work and find additional factors that may be carried over from the round spermatid. As this has direct relevance for the assisted reproduction field, it is of likely to be of high significance for the field.

Reviewer 2 Comments for the author
The authors have addressed all of my prior concerns. Their revised manuscript incorporates stronger statistical analyses to support their claims and has a more focused narrative that makes the overall story easier to follow. I appreciate their work that attempted to understand the differences in the active regions via studying other chromatin marks and transcription factors. I do not think any further revisions are required.

Response: We would like to thank all the constructive comments from this reviewer, especially the suggestions for data quantification.

Reviewer 3 Advance summary and potential significance to field
These issues have been described at the initial review.

Reviewer 3 Comments for the author
In the revised manuscript, the authors have addressed satisfactorily some of the issues raised in the initial review. However, some serious problems still remain as described below.
The purpose of this study is to elucidate the mechanism causing a low birth rate in ROSI-embryos. However, this manuscript fails in providing a plausible mechanism for it. Although the authors provide the results of analyzing various factors involved in the developmental process, e.g. gene

expression, epigenetic modifications and open chromatin sites, they fail in elucidating the relationships among these factors to construct a plausible mechanism. Although the gene expression pattern is different between ROSI- and ICSI-embryos, which seems to be a cause of a low birth rate in ROSI-embryos, the mechanism underlying this difference is not elucidated. In the hypothesis shown in Fig. 6, the genes with H3K27me3 in round spermatids keep this modification at the 1- and 2-cell stages after ROSI. In these embryos, H3K27me3 causes the lower accessibility of chromatin, leading to the suppression of gene expression. However, only a small part of ROSI-DEGs is identical between 1- and 2-cell (Fig. S2C). Furthermore, most of ROSI-high and low peaks in ATAC analysis are different between 1- and 2-cell (Fig. S3B). These results indicate that the chromatin accessible state and increased expression of a gene is not kept from 1-cell to 2-cell, which is inconsistent with the hypothesis in Fig. 6. Finally, Fig. 2E shows that the mRNAs derived from round spermatids are introduced into ROSI-1 cell embryos. In this case, it seems to make no sense to analyze open chromatin and epigenetic modifications in ROSI-1-cell embryos to elucidate the causes of ROSI-high DEGs. Therefore, the manuscript should be thoroughly revised.

Response:
We thank the reviewer for these comments and apologize for the lack of enough explanation for the data interpretation.

In this study, we identified the promoter regions which showed lower accessibility in ROSI 1-cell embryos (the genomic regions shown in Fig. 6). Further analysis revealed that H3K27me3 is deposited at these regions in round spermatids. Comparison of the ATAC-seq data with RNA-seq data indicated that expression of the genes associated with these promoters are slightly downregulated in ROSI 2-cell embryos but not in ROSI 1-cell embryos. However, the extent of the downregulation was mild, and they were not detected as DEGs (as described in Fig. 5B). Therefore, DEGs are not the subject of our discussion in Fig. 6. Nevertheless, one might expect that ROSI 1-cell embryos would also show defective expression of these genes, but it was not the case. However, this is expected as mouse embryos at the 1-cell stage have extremely limited transcriptional activity compared to embryos at the 2-cell stage. In other words, it is hard to detect transcriptional alteration in ROSI 1-cell embryos by a conventional RNA-seq technique even if changes in chromatin accessibility affect transcription. Although we already discussed these possibilities in Discussion (line 296-300), we apologize that these explanations are not clearly indicated in our model in Fig. 6. Thus, we changed the figure legend for Fig. 6 where possible changes in transcription at the 1-cell stage are now described (line 820-832).

It is true that “most of ROSI-high and low peaks in ATAC analysis are different between 1- and 2-cell (Fig. S3B)”, and altered chromatin accessibility upon ROSI application was highly represented in 1-cell ROSI-embryos (Fig. S3B). In contrast to our expectation, at the genomic regions indicated in Fig. 6, chromatin accessibility was not different between ROSI and ICSI embryos at the 2-cell stage when changes in gene expression were detected. Thus, one might consider that the altered chromatin accessibility has nothing to do with gene expression. However, our closer examination revealed that lowered chromatin accessibility clearly preceded the downregulation of gene expression. Thus, our model in Fig.6 summarizes these observations and indicates that chromatin accessible state is not necessarily kept from 1-cell to 2-cell to change gene expression. As we think that our explanation was not sufficient in our previous manuscript, we included an additional explanation in the Discussion (line 306-310) to facilitate the understanding of the proposed model in Fig. 6.

With technical limitations described above, we understand that it is hard to detect clear relationship between chromatin accessibility and transcription at the 1-cell stage. Nevertheless, we performed ATAC-seq and RNA-seq using ROSI and ICSI 1-cell and 2-cell embryos to sufficiently prepare the datasets for ROSI and ICSI embryos and to deepen our understanding of biological differences between those embryos. We totally agree with the opinion that “It seems to make no sense to analyze open chromatin and epigenetic modifications in ROSI-1-cell embryos to elucidate the causes of ROSI-high DEGs.”, and therefore we simply concluded that the main cause of 1-cell ROSI DEGs is the inheritance of RNAs from round spermatids. We have no intention of relating these DEGs to changes in chromatin accessibility.

In Fig. 3SD, the quantitative analysis should be conducted for H3K27me3 as well as H3K4me3 (H3K27me3 is rather more important).

Response: We provide the quantified results in Fig. S3D.

Third decision letter

MS ID#: DEVELOP/2022/200696

MS TITLE: Paternally inherited H3K27me3 affects chromatin accessibility in mouse embryos produced by round spermatid injection

AUTHORS: Mizuki Sakamoto, Daiyu Ito, Rei Inoue, Sayaka Wakayama, Yasuyuki Kikuchi, Li Yang, Erika Hayashi, Rina Emura, Hirosuke Shiura, Takashi Kohda, Satoshi H Namekawa, Takashi Ishiuchi, Teruhiko Wakayama, and Masatoshi Ooga
ARTICLE TYPE: Research Article

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