# The microRNA miR-202 prevents precocious spermatogonial differentiation and meiotic initiation during mouse spermatogenesis

Jian Chen, Chenxu Gao, Xiwen Lin, Yan Ning, Wei He, Chunwei Zheng, Daoqin Zhang, Lin Yan, Binjie Jiang, Yuting Zhao, Md Alim Hossen and Chunsheng Han
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**Review timeline**

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**Original submission**

First decision letter

MS ID#: DEVELOP/2021/199799

MS TITLE: MicroRNA-202 prevents precocious spermatogonial differentiation and meiotic initiation during mouse spermatogenesis

AUTHORS: Jian Chen, Chenxu Gao, Xiwen Lin, Yan Ning, Wei He, Chunwei Zheng, Daoqin Zhang, Lin Yan, Binjie Jiang, Yuting Zhao, Md Alim Hossen, and Chunsheng Han

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. There are several issues that would need to be addressed. Better characterisation of the miRNA-202 deletion is required to determine which cells are affected. Is the deletion limited to germ cells or are Sertoli cells (or other somatic cells) also targeted, and does this alter the interpretation of the data? Reviewer 2 raises questions about the purity of the cells isolated using STAPUT that should be addressed. All three reviewers also highlight several additional issues that need to be addressed and will require clarifications or further analyses. In addition, Reviewer 1 asks about the reproductive performance of the mutant mice and whether there is age-related infertility. Finally, I would draw your attention to the question raised by Reviewer 2 about the Western blots presented in Fig S9B.

If you are able to address the concerns raised by the referees, which may involve further experiments, I will be happy to 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.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

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 manuscript demonstrates a specific role for miRNA-202 and controlling the timing of spermatogonial differentiation and entry into meiosis. The work provides insight into the temporal control of spermatogenesis, of which we know surprisingly little.

*Comments for the author*

In this manuscript, Chen et al examine the effects of deleting miRNA-202 on mouse spermatogenesis. They find that miRNA-202 ablation in mice and cultured spermatogonial stem cells (SSCs) causes depletion of undifferentiated spermatogonia (SGundiff) and premature entry of germ cells into meiosis, ultimately leasing to agametic tubules. Using additional genetic manipulation experiments in SSCs, they posit a mechanism by which miRNA-202 performs these roles via regulation of Dmrt6.
These are a series of well-designed and executed experiments, and the phenotypes are robust and interesting. We had the following comments:

Major revisions:
1. It is surprising that the authors did not assess the reproductive performance of miRNA-202 mutant males; is there age-related infertility?

2. The nature of the miRNA-202 disruption is not discussed in appropriate detail. The authors refer to a preprint, but the targeting information should be included in this submission. Even in the preprint, the details are too rudimentary for objective assessment. The CRISPR-Cas9 targeting approach and sequence details need to be added to the supplementary material.

3. Further clarification is needed regarding the spermatogonial phenotypes.

It is currently unclear whether the SGundiff pool is perturbed from the outset or is depleted over time. In the first results paragraph, the authors argue that “MiR-202 knockout reduces the undifferentiated spermatogonial pool”. It is however unclear whether the KO mice start with normal numbers of SGundiff or whether the number of SGundiff is already reduced in the first wave of spermatogenesis. Further characterisation of KO mice is needed during the first wave of spermatogenesis. It could be that the KO mice start with the same amount of SGundiff or that the number is already lower and becomes more apparent as the mice age and exhaust the stem cell pool. If the mice start with similar numbers of SGundiff, then the stem cell pool is not reduced in KO mice. Instead, the pool is exhausted more rapidly. Finally, it is unclear as of why some of these experiments are performed at 4months and others at 12 months of age.

4. Related to point 3 above, the data on the differentiating spermatogonia also need further clarification. In the first paragraph of the results, the authors describe that in 4-month-old mice: “the numbers of differentiating spermatogonia represented by the KIT+ cells inside the tubules were similar in the KO and WT mice”. This finding is at odds with the rest of the paper which describes increased numbers of differentiating spermatogonia and meiotic cells in the KO model.

This statement also contradicts the findings in vitro: “one day after induction, we found that 18% of the KO cells started to express KIT, whereas only 7% WT cells did so”. Wouldn’t you expect higher proportions of KIT+ cells in the KO mouse?

Minor revisions:
1. In order to be fully transparent, it may be worth mentioning in the paper that MiRNA-202 lies within the lncRNA Gm2044-201. As a result, the deletion of mir-202 may also perturb lncRNA Gm2044-201 expression. The rescue of the phenotypes using the mir-202 mimic does seem to indicate that the phenotypes are a result of mir-202 KO and not other perturbations. However, it would be reassuring to see data indicating that lncRNA Gm2044-201 is not mis-regulated in KO mice.
2. line 32: “many genes including those for other key regulators”: a bit vague could be re-phrased.
3. line 45: change “huge”.
4. line 60-61: “which is the hallmark of gametogenesis”: Remove.
5. line 91: transition between line 91 and 92 feels abrupt. The 2 paragraphs are not well linked.
6. line 126: “some tubules”: give a percentage to be more specific.
7. line 139-144: Could it be that the KO sample had more contamination of later-stage germ cells, therefore explaining enrichment in later-stage spermatogenesis markers? Showing the fraction purity of sequenced KO and WT samples would help addressing this possibility. The authors cite the purity as >85%, but it isn’t clear whether the remaining 15% are comprised of similar cell types in the KO vs WT.
8. line 151-153: I find this sentence too strong. The only evidence definitively demonstrating that precocious spermatogonial differentiation and meiotic entry are occurring is the cytological data that appear later in the paper.
9. line 175-176: Please rephrase this first sentence. Co-immunostaining tells you “how” an increased gene expression arises.
10. paragraph starting with line 186: This paragraph refers to pre-activation of DMRT6. It may not be appropriate to use the term “pre-activation” in that context. The immunostaining work shows earlier expression of DMRT6, but it doesn’t show earlier activity/function. Pre-activation implies a mechanistic aspect not shown in the paragraph. Maybe re-phrase to : “DMRT6 expression is premature in the absence of miR-202.”
11. line 199-202: this section is a bit confusing for audience with little knowledge on luciferase assays. The experimental approach and results need unpacking.
12. line 206 and S5: Please make it clearer that the idea of this experiment is to show that KO effect is independent of DMRT1.
13. line 243-244: this contradicts the finding in 4-month-old KO that have the same ration of KIT+ cells. See major comments.
14. Fig1B: Authors wrote NVH instead of MVH.
15. Fig1D: the difference between the KO and WT is not very clear in this tubule section. Maybe using another image would help tell the story better?
16. Fig3A: These panels are quite hard to interpret and the zoom in inserts are very small. Maybe the quantification data would be enough for the main figure and the immunostaining could be added to supp materials.
17. Fig3C: These panels are quite busy and a bit hard to interpret. Maybe it would be better to zoom on a single tubule for each stage (i.e one panel for I-IV, one for VII-VIII, one for IX-XII). This would make it easier the immunostaining signals.
18. Fig5: No quantification data is provided here. It would be useful to have bar charts like seen in Fig3 for instance.

Reviewer 2

*Advance summary and potential significance to field*

In this work, the authors explore the phenotype of miR-202 KO mice. This reviewer has multiple conceptual problems and technical concerns that made interpreting the results difficult. The potential significance to the field is difficult to ascertain due to these significant conceptual and technical issues.

*Comments for the author*

First - the results here posit a critical role for a specific miRNA in regulating spermatogonial function. However, this is hard to understand, as mice with germline deletion of Dicer using Ddx4-Cre (active as early as E15.5 in prospermatogonia/gonocytes) which presumably lack ALL miRNAs did not have a spermatogonial defect, but rather a meiotic one (PMID: 21998645); Using Tnap-Cre (which is likely activated earlier, in PGCs) – those mice had sperm, but more Sertoli cell only tubules; Using logic to compare those 2 studies, it seems that the loss of premeiotic germ cells in the Tnap-Cre model may be due to effects on the PGC population; Also, the BCAS2 KO mice have meiotic defects and normal spermatogonia populations (PMID: 28128212); These specifics should be included in the introduction – the mouse infertility in those mice seems to be due to a meiotic defect and not a spermatogonial one (e.g. PMID: 25244517, 25934699); Also, speaking to the conservation of such a broad regulatory program (mentioned in lines 92-94), Zebrafish with Dicer1 mutation had no germline defects (PMIDs: 17418787, 15774722)… So, how does loss of this single miRNA have such a profound effect, while its loss with all the others apparently had none?
Second - the spermatogonial isolations for RNA-seq are hugely problematic. A careful analysis of the methods (413-426) reveals a major concern. STAPUT isolates cells based on size alone. The most undifferentiated type A spermatogonia MAY be significantly large enough to separate from somatic cells, but certainly other spermatogonia (differentiating A1-4, In, and B) that are present in the P6-7 testis will not be. In Fig. S3, immunostaining was done using DMRT1 – problem is it's also well-known to be expressed in Sertoli cells. So, Fig. S2B is not at all a confirmation of germ cell purity, as you cannot discern Sertoli from germ based on the stain. So it is completely unclear to this reviewer what cells were used for RNA-seq. Some were certainly germ cells, as the authors picked up germ cell mRNAs, but the purity is unknown, and therefore it is difficult to impossible to make any conclusions whatsoever about the data. Should do scRNA-seq, which does not require cell isolation, and could compare to published reports on those ages.
Third - the immunostaining results are perplexing in Fig 2 - the staining patterns for SYCP3 and STRA8 in WT mice do not look like this; since the first wave of spermatogenesis proceeds in a predictable fashion, cord cross-sections either have STRA8+ spg or preleps (lots of them) or they don't. Same with SYCP3. It's not expected to see 1, or 2 positive cells like that. Also, why the interstitial staining for STRA8? It's a germ cell specific protein
Fourth - if there is a consistent loss of the undifferentiated population, wouldn't spermatogenesis consistently decline? Why then in Fig. S1 is only one abnormal tubule shown next to completely normal-appearing ones? And no problems until 12 months?? The tubule indicated by the asterisk resembles tubuli recti, or straight tubules, which connect the seminiferous tubules to the rete testis and typically lack germ cells;
Fifth - there are too many errors and inconsistencies that make this reviewer question the validity of the results. Fig. S1 - MVH is not at all a Sertoli cell marker, but expressed in germ cells; Fig. S3 - DMRT1 is expressed in Sertoli and germ cells, so cannot be used to determine germ cell purity after isolation; Fig. S4 - why so much interstitial staining for germ cell markers? And the staining results do not match expectations for the biology of the first wave of spermatogenesis, as described above; Fig. S5 - why is DMRT1 not showing up in Sertoli cells?? And why is PLZF showing up in the interstitium? Fig. S9B - those look like the exact same blots, for STRA8 and DMRT6! And isn't ACTB more like 42 kDa - so why the difference between A and B? DMRT6 has 2 isoforms, 22 and 38 kDa - so why is it showing up at the same spot as STRA8? STRA8 is 37 kDa, so why running so high? This series of western blots (which are not "uncropped", as the title suggests, is a big concern to this reviewer.

Reviewer 3

*Advance summary and potential significance to field*

This is an interesting and insightful work which mainly focused on the regulation network on meiotic initiation. The authors showed that miR-202, a member of the let-7 family, prevents precocious spermatogonial differentiation and meiotic initiation in spermatogenesis. Knockout of miR-202 will lead to the reduction of undifferentiated spermatogonial pool and ultimately causing agametic seminiferous tubules through the earlier spermatogonial differentiation and meiotic initiation. While the molecular assays explored the working mechanism of miR-202 in spermatogenesis, which showed that miR-202 directly targets Dmrt6 mRNA and restricts the

expression window of DMRT6 in SGdiff, to coordinate an orderly transition from the mitosis to the meiosis. The in vivo and in vitro results could well support the conclusion.

*Comments for the author*

there are several minor issues need to be addressed.
1. How is the expression pattern of miR-202 in testis, does it localize in Sertoli cell and affect Sertoli cell functions?
2. the in vitro experiments showed the increased apoptosis in ko group, how is going in vivo ?
3. the authors showed knockout miR-202 lead to advanced differentiation of SG and exhaustion of SG pool. Will it affect SG proliferation?
4. In the discussion, it concludes that knockout of miR-202 phenocopy mutant of DMRT1, NANOS2, MAX and AGO4, does miR-202 also target these genes?
5. line 225: there are two "in"

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**First revision**

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
This manuscript demonstrates a specific role for miRNA-202 and controlling the timing of spermatogonial differentiation and entry into meiosis. The work provides insight into the temporal control of spermatogenesis, of which we know surprisingly little.

Reviewer 1 Comments for the Author:
In this manuscript, Chen et al examine the effects of deleting miRNA-202 on mouse spermatogenesis. They find that miRNA-202 ablation in mice and cultured spermatogonial stem cells (SSCs) causes depletion of undifferentiated spermatogonia (SGundiff) and premature entry of germ cells into meiosis, ultimately leasing to agametic tubules. Using additional genetic manipulation experiments in SSCs, they posit a mechanism by which miRNA-202 performs these roles via regulation of Dmrt6.
These are a series of well-designed and executed experiments, and the phenotypes are robust and interesting. We had the following comments:

Major revisions:
1. It is surprising that the authors did not assess the reproductive performance of miRNA-202 mutant males; is there age-related infertility?
**Response:** The reproductive performance of KO mice was examined and reported in a separate manuscript, of which a copy can be accessed through https://doi.org/10.1101/2021.04.14.439735 in bioRxiv. In short, we found that the fertility of the KO mice was reduced. Moreover, we found that the fertility was reduced in an age-dependent manner, and this observation has been added to the revised version of this manuscript (Fig. 1C).

2. The nature of the miRNA-202 disruption is not discussed in appropriate detail. The authors refer to a preprint, but the targeting information should be included in this submission. Even in the preprint, the details are too rudimentary for objective assessment. The CRISPR-Cas9 targeting approach and sequence details need to be added to the supplementary material.
**Response:** We are sorry for this problem. We have added more details for knockout approach including sequences of *miR-202* gene locus before and after gene KO to the above manuscript (https://doi.org/10.1101/2021.04.14.439735). A description has been added to the Materials and Methods section of this revised manuscript.

3. Further clarification is needed regarding the spermatogonial phenotypes. It is currently unclear whether the SGundiff pool is perturbed from the outset or is depleted over time. In the first results paragraph, the authors argue that “MiR-202 knockout reduces the undifferentiated spermatogonial pool”. It is however unclear whether the KO mice start with normal numbers of SGundiff or whether the number of SGundiff is already reduced in the first wave of spermatogenesis. Further

characterisation of KO mice is needed during the first wave of spermatogenesis. It could be that the KO mice start with the same amount of SGundiff or that the number is already lower and becomes more apparent as the mice age and exhaust the stem cell pool. If the mice start with similar numbers of SGundiff, then the stem cell pool is not reduced in KO mice. Instead, the pool is exhausted more rapidly. Finally, it is unclear as of why some of these experiments are performed at 4months and others at 12 months of age.
**Response:** We thank the reviewer very much for this important question. We have examined the $PLZF^+$ undifferentiated spermatogonia in mice at postnatal day 9 (P9) and found that the numbers of SGundiff were comparable between KO and WT mice at this time. This observation together with that SGundiff were reduced by 43% in the adult KO mice indicate that *miR-202* KO resulted in the exhaustion of the SGundiff pool in adults but had no effect on its establishment after birth. The new results have been added to the first paragraph of the results section (Fig. 1C).
The fertility of the KO mice starts to drop since 4 months after birth (Fig. 1B). And the morphological defects become apparent since 4 months and were the most apparent at 12 months. So, we just used the morphological data from KO mice of 12 months in the beginning and then used the 4-month mice to generate other data for adult mice.

4. Related to point 3 above, the data on the differentiating spermatogonia also need further clarification. In the first paragraph of the results, the authors describe that in 4- month-old mice: "the numbers of differentiating spermatogonia represented by the KIT+ cells inside the tubules were similar in the KO and WT mice". This finding is at odds with the rest of the paper which describes increased numbers of differentiating spermatogonia and meiotic cells in the KO model. This statement also contradicts the findings in vitro: "one day after induction, we found that 18% of the KO cells started to express KIT, whereas only 7% WT cells did so". Wouldn't you expect higher proportions of KIT+ cells in the KO mouse?

**Response:** We agree with the reviewer that the number of $KIT^+$ cells was not changed in adult KO mice is kind of surprising. However, this is not necessarily at odds with the rest of the paper which describes the premature initiation of both spermatogonial differentiation and meiosis. Please note that we only reported the increased numbers of $SYCP3^+$ meiotic cells and $STRA8^+$ spermatogonial at P9 which represent the first wave of spermatogenesis in the KO model. If both spermatogonial differentiation and meiotic initiate precociously in KO mice, it is possible that the net effect is the unchanged number of $KIT^+$ cells in adult mice. However, for the first wave spermatogenesis, the number of $STRA8^+$ spermatogonia can be accumulated to some degree as the meiosis just initiates at P9. These explanations have been added to the discussion section.

Minor revisions:
1. In order to be fully transparent, it may be worth mentioning in the paper that MiRNA-202 lies within the lncRNA Gm2044-201. As a result, the deletion of mir-202 may also perturb lncRNA Gm2044-201 expression. The rescue of the phenotypes using the mir-202 mimic does seem to indicate that the phenotypes are a result of mir- 202 KO and not other perturbations. However, it would be reassuring to see data indicating that lncRNA Gm2044-201 is not mis-regulated in KO mice.
**Response:** We thank the reviewer for this good suggestion. As shown by Fig. S1D, the expression level of Gm2044-201 in isolated SG-A is not changed in KO mice. We have added this result in the results section (lines 163-166).

2. line 32: "many genes including those for other key regulators": a bit vague could be re-phrased.
**Response:** The sentence has been rephrased to "many genes including those for key regulators such as STRA8 and DMRT6".

3. line 45: change "huge".
**Response:** We have changed "huge" to "big".

4. line 60-61: "which is the hallmark of gametogenesis": Remove.
**Response:** Done.

5. line 91: transition between line 91 and 92 feels abrupt. The 2 paragraphs are not well linked.

**Response:** We have rephrased the writing, please see lines 91-96.

6. line 126: “some tubules”: give a percentage to be more specific.
**Response:** We have counted the abnormal tubules and the quantification has been added to the text and as a new figure (Fig 1A).

7. line 139-144: Could it be that the KO sample had more contamination of later-stage germ cells, therefore explaining enrichment in later-stage spermatogenesis markers? Showing the fraction purity of sequenced KO and WT samples would help addressing this possibility. The authors cite the purity as >85%, but it isn’t clear whether the remaining 15% are comprised of similar cell types in the KO vs WT.
**Response:** We isolated the SG-A by STAPUT approach, which is commonly used for isolating mouse spermatogenic cells for omic studies. According to a previous study from our group, the purity is at least 85% (Gan et al., 2013a). To assess the purify of isolated SG-A in this study, we have performed the immunostaining of PLZF, which is the marker for SGundiff, on pooled fractions of the isolated SG-A, and the purity was 92-94% (Fig. S3A). This explanation has been added to the Materials and Methods section.

8. line 151-153: I find this sentence too strong. The only evidence definitively demonstrating that precocious spermatogonial differentiation and meiotic entry are occurring is the cytological data that appear later in the paper.
**Response:** We quite agree with the reviewer with this point. The sentence has been modified as “These results suggested that the reduction of SGundiff pool might be caused by the aberrant expression of differentiation- and meiosis-related gene”.

9. line 175-176: Please rephrase this first sentence. Co-immunostaining tells you “how” an increased gene expression arises.
**Response:** We have changed the sentence to “We next examined whether STRA8 was precociously expressed in SGundiff by co-immunostaining for STRA8 and PLZF” in lines 187-188.

10. paragraph starting with line 186: This paragraph refers to pre-activation of DMRT6. It may not be appropriate to use the term “pre-activation” in that context.
The immunostaining work shows earlier expression of DMRT6, but it doesn’t show earlier activity/function. Pre-activation implies a mechanistic aspect not shown in the paragraph. Maybe re-phrase to : “DMRT6 expression is premature in the absence of miR-202.”
**Response:** Thanks and we have changed the description.

11. line 199-202: this section is a bit confusing for audience with little knowledge on luciferase assays. The experimental approach and results need unpacking.
**Response:** We have expanded the description of the assay into a paragraph in the result section (lines 208-213) and also added more details in the Materials and Methods section (lines 452-457).

12. line 206 and S5: Please make it clearer that the idea of this experiment is to show that KO effect is independent of DMRT1.
**Response:** OK, the writing has been modified and moved to the beginning of the section (lines 200-203).
13. line 243-244: this contradicts the finding in 4-month-old KO that have the same ration of KIT+ cells. See major comments.
**Response:** Please see our explanation to the major comment 4.

14. Fig1B: Authors wrote NVH instead of MVH.
**Response:** Corrected. Thank you very much.

15. Fig1D: the difference between the KO and WT is not very clear in this tubule section. Maybe using another image would help tell the story better?
**Response:** Yes, we have replaced the old images.

16. Fig3A: These panels are quite hard to interpret and the zoom in inserts are very small. Maybe the quantification data would be enough for the main figure and the immunostaining could be

added to supp materials.

**Response:** We have labeled the images with arrows and arrowheads representing $STRA8^-$ and $STRA8^+$ in PLZF positive cells so that they are easier to interpret. We have removed the zoom-in insets as they do not contain extra details.

17. Fig3C: These panels are quite busy and a bit hard to interpret. Maybe it would be better to zoom on a single tubule for each stage (i.e one panel for I-IV, one for VII- VIII, one for IX-XII). This would make it easier the immunostaining signals.
**Response:** We thank the reviewer for these great suggestions. We have changed the images according to the suggestions.

18. Fig5: No quantification data is provided here. It would be useful to have bar charts like seen in Fig3 for instance.
**Response:** We have added the bar charts for statistics.

Reviewer 2 Advance Summary and Potential Significance to Field:
In this work, the authors explore the phenotype of miR-202 KO mice. This reviewer has multiple conceptual problems and technical concerns that made interpreting the results difficult. The potential significance to the field is difficult to ascertain due to these significant conceptual and technical issues.

Reviewer 2 Comments for the Author:
First - the results here posit a critical role for a specific miRNA in regulating spermatogonial function. However, this is hard to understand, as mice with germline deletion of Dicer using Ddx4-Cre (active as early as E15.5 in prospermatogonia/gonocytes) which presumably lack ALL miRNAs did not have a spermatogonial defect, but rather a meiotic one (PMID: 21998645); Using Tnap-Cre (which is likely activated earlier, in PGCs) – those mice had sperm, but more Sertoli cell only tubules; Using logic to compare those 2 studies, it seems that the loss of premeiotic germ cells in the Tnap-Cre model may be due to effects on the PGC population; Also, the BCAS2 KO mice have meiotic defects and normal spermatogonia populations (PMID: 28128212); These specifics should be included in the introduction – the mouse infertility in those mice seems to be due to a meiotic defect and not a spermatogonial one (e.g. PMID: 25244517, 25934699); Also, speaking to the conservation of such a broad regulatory program (mentioned in lines 92-94), Zebrafish with Dicer1 mutation had no germline defects (PMIDs: 17418787, 15774722)… So, how does loss of this single miRNA have such a profound effect, while its loss with all the others apparently had none?
**Response:** We thank the reviewer very much for this good question “how does loss of this single miRNA have such a profound effect, while its loss with all the others apparently had none?” After critical thinking, we found that this question contains one assumption and one interesting question. The assumption is that *miR-202* KO has a profound effect while the KO of all miRNAs as a result of the KO of *Dicer* had none. As the reviewer pointed out the KO of *Dicer* resulted in defects in both meiocytes and PGCs while the KO of *miR-202* resulted in defects in spermatogonia but not in PGCs, it is hard to say that *miR-202* has a profound effect but its loss with all the others apparently had none. More importantly, Romero *et al*. (Romero et al., 2011) also reported a time-dependent decrease in testis weight and increase in apoptotic cells similar to the time-dependent spermatogenic failure in our *miR-202* KO mice, which implied a problem in the spermatogonial proliferation phase of spermatogenesis.

Unfortunately, they stopped their observation by P180. Two more studies (Zimmermann et al., 2014; Modzelewski et al., 2015) reported similar but slightly different phenotypes between *Dicer* and *Dgcr8* KO mice. Again, their observations did not last beyond P60 and P70, respectively. In our study, abnormal morphology in testis sections is not apparent by histological evaluation until 4 months after birth and the defects in spermatogonia can only be disclosed by co-immunostaining and careful inspection of marker proteins such as PLZF, STRA8, DMRT6 and SYCP3. The previous studies failed to conduct these in-depth analyses. Therefore, it is more accurate to say that *miR-202* plays a role in spermatogonia but this role is not very critical because the loss of spermatogonia is partial and gradual. In contrast, tubules containing few or none germ cells were readily observed in the *Tnap*-Cre/*Dicer* KO mice 4 weeks after birth (Hayashi et al., 2008). This suggest that PGC formed in the beginning but got lost at a later stage, suggesting a role of *Dicer* in spermatogonial phase. Consistently, the authors reported that the *Dicer* KO spermatogonia can be

cultured *in vitro* for 2 weeks with reduced capacity for proliferation. Therefore, it is very likely Dice also plays a role in spermatogonial proliferation.

The interesting question is “why the effect of a part of a system (*miR-202*) is bigger than the whole (all miRNAs represented by Dicer)?” This may be answered by using a weighing balance as a metaphor of the miRNA system. The DICER is like the gravity field that applies weights to all miRNA objects in the two plates of the balance. The balance can always be maintained balanced no matter how strong the gravity field is, even when it is not there, but not when a single object in any of the plates is removed. In this sense, the KO of *miR-202* may have a more profound effect than the KO of *Dicer*, although this may not be true as pointed out above.

The reviewer also pointed out that BCAS2 KO mice have meiotic defects and normal spermatogonia populations (Liu et al., 2017). As we know that BCAS2 is involved in mRNA splicing and we do not how it is related to the miRNA system, we are not sure how this example helps us to understand the functions and mechanisms of the miRNA system. The reviewer also pointed out that zebrafish with *Dicer1* mutation had no germline defects (Giraldez et al., 2005; Houwing et al., 2007). This argument only shows that the function of *Dicer* in gametogenesis is not evolutionarily conserver because that *Dicer* has a role in mammalian gametogenesis is a fact confirmed by several independent studies. We find that this fact does not help us to understand the seemingly discrepant phenotypes of the *Dicer1* and *miR-202* KO mice.

As another piece of evidences for a role of miRNAs in spermatogonia, Tong *et al*. have reported that two miRNA Clusters, Mir-17-92 (Mirc1) and Mir-106b-25 (Mirc3), are involved in the regulation of spermatogonial differentiation in mice (Tong et al., 2012).

Again, we thank the reviewer very much for this stimulating question and we have discussed this issue in the revised manuscript (lines 304-319).

Second - the spermatogonial isolations for RNA-seq are hugely problematic. A careful analysis of the methods (413-426) reveals a major concern. STAPUT isolates cells based on size alone. The most undifferentiated type A spermatogonia MAY be significantly large enough to separate from somatic cells, but certainly other spermatogonia (differentiating A1-4, In, and B) that are present in the P6-7 testis will not be. In Fig. S3, immunostaining was done using DMRT1 – problem is it’s also well-known to be expressed in Sertoli cells. So, Fig. S4B is not at all a confirmation of germ cell purity, as you cannot discern Sertoli from germ based on the stain. So it is completely unclear to this reviewer what cells were used for RNA-seq. Some were certainly germ cells, as the authors picked up germ cell mRNAs, but the purity is unknown, and therefore it is difficult to impossible to make any conclusions whatsoever about the data. Should do scRNA-seq, which does not require cell isolation, and could compare to published reports on those ages.
**Response:** We are sorry for mistakenly using DMRT1 as a marker for SG-A. We have used the STAPUT method to enrich different spermatogenic cells in several omic studies (Gan et al., 2011; Gan et al., 2013a; Gan et al., 2013b; Lin et al., 2016) and this technique in our hand is mature and stable. In this study, the cell types and purity in each fraction were initially assessed using a light microscope based on their diameters and morphological characteristics. Fractions highly enriched in SG-A based on the morphological evaluation were pooled and the purify was assessed by immunostaining of marker proteins. In the revised manuscript, we have used immunostaining of PLZF, which is a marker of SGundiff, to show that the enriched SG- A are mostly $PLZF^{+}$ SGundiff, and the purity is 92-94% (Fig. S3A).
To provide more evidence, we compiled a list of genes expressed in spermatogenic cells based on one of our previous studies (Lin et al., 2016) and a list of genes expressed in $Thy1^{+}$ undifferentiated SG-A based on a study by others (Maezawa et al., 2020). The intersection between these two lists of genes contains 13690 genes. We have found that 88% of our upregulated genes and 94% of our downregulated genes in the *miR-202* KO mice are in this13,690 intersection gene list. This result supports that most of the differentially expressed genes (DEGs) are from the isolated SG. This result has been added to lines 152-157. We have also tried the scRNA-seq method, unfortunately, we were unable identify DEGs between KO and WT SG-A probably due to the shallow sequencing depth of this technique as well as the week effect of *miR-202* on gene expression in this cell type. We identified a small number of DEGs in spermatocytes and the result was added to the other manuscript but not this one (Chen et al., 2021 preprint).

Third - the immunostaining results are perplexing in Fig 2 - the staining patterns for SYCP3 and STRA8 in WT mice do not look like this; since the first wave of spermatogenesis proceeds in a predictable fashion, cord cross-sections either have STRA8+ spg or preleps (lots of them) or they don't. Same with SYCP3. It's not expected to see 1, or 2 positive cells like that. Also, why the interstitial staining for STRA8? It's a germ cell specific protein
**Response:** Again, we are sorry for these low quality images due to our technical insufficiency. We have repeated the immuostainings of SYCP3 in P9 testis and STRA8 in P9 and adult testes. We found that the P9 testes indeed contain more $SYCP3^{+}$ and $STRA8^{+}$ cells than the previous image showed. The lower numbers of such cells in the previous images were due to a weaker staining, and the experiments have been repeated and new images have been provided. The nonspecific interstitial staining for STRA8 has also been removed by using a different secondary antibody.

Fourth - if there is a consistent loss of the undifferentiated population, wouldn't spermatogenesis consistently decline? Why then in Fig. S1 is only one abnormal tubule shown next to completely normal-appearing ones? And no problems until 12 months?? The tubule indicated by the asterisk resembles tubuli recti, or straight tubules, which connect the seminiferous tubules to the rete testis and typically lack germ cells;
**Response:** Yes, as the reviewer predicted, we have found that the fertility of the KO mice drops in an age-dependent manner (Fig. 1B), and we have also observed tubules containing few undifferentiated spermatogonia in mice in 4 months after birth (Fig. 1C). These results indicate the consistent loss of the undifferentiated spermatogonial population. However, we need to point out that spermatogonial loss is partial as tubules that are either agametic or almost agametic are sporadic among tubules that are defective to different degrees. We did not observe agametic tubules in mice of 8 months probably due to the infrequent occurring of such severely defective tubules. The reviewer questioned whether the agametic tubule shown in the original Fig. S1B is a tubulus rectus (plural tubuli recti). We think it is not because tubuli recti connect the seminiferous tubules to the rete extensions and therefore they are usually seen together with sections of the rete testis (Osman and Ploen, 1978). As can be seen from the original Fig. S1B, this questionable tubulus is surrounded by normal looking sections from the seminiferous tubules but not special sections from rete testis.
Despite of this argument, to eliminate any possible confusion, we have replaced the original image with new ones to show the occurrence of such tubules in different locations (Fig. 1A). We also added a new image to show that most of the tubules at 12 months are abnormal (Fig. S1B).

Fifth - there are too many errors and inconsistencies that make this reviewer question the validity of the results. Fig. S1 - MVH is not at all a Sertoli cell marker, but expressed in germ cells; Fig. S3 - DMRT1 is expressed in Sertoli and germ cells, so cannot be used to determine germ cell purity after isolation; Fig. S4 - why so much interstitial staining for germ cell markers? And the staining results do not match expectations for the biology of the first wave of spermatogenesis, as described above;
Fig. S5 - why is DMRT1 not showing up in Sertoli cells?? And why is PLZF showing up in the interstitium? Fig. S9B - those look like the exact same blots, for STRA8 and DMRT6! And isn't ACTB more like 42 kDa - so why the difference between A and B? DMRT6 has 2 isoforms, 22 and 38 kDa - so why is it showing up at the same spot as STRA8? STRA8 is 37 kDa, so why running so high? This series of western blots (which are not "uncropped", as the title suggests, is a big concern to this reviewer.
**Response:** We are sorry for those careless mistakes. The following are our point-by- point responses to these questions.

Fig. S1 - MVH is not at all a Sertoli cell marker, but expressed in germ cells;
**Response:** We mis-spelled WT1 as MVH in the legend of this figure. Corrected.

Fig. S3 - DMRT1 is expressed in Sertoli and germ cells, so cannot be used to determine germ cell purity after isolation;
**Response:** Explained in response to the second major concern.

Fig. S4 - why so much interstitial staining for germ cell markers? And the staining results do not match expectations for the biology of the first wave of spermatogenesis, as described above;

**Response:** Explained in our response to the third major concern.

Fig. S5 - why is DMRT1 not showing up in Sertoli cells?? And why is PLZF showing up in the interstitium?
**Response:** DMRT1 can be seen in Sertoli cells in the original images but the signal is much weaker than in germ cells. We have replaced the old image with a new one (Fig. S5). But the difference in signal intensity between Sertoli cells and germ cells is still apparent. The nonspecific staining of PLZF in the interstitium has been eliminated by using a different secondary antibody.

Fig. S9B - those look like the exact same blots, for STRA8 and DMRT6! And isn't ACTB more like 42 kDa - so why the difference between A and B? DMRT6 has 2 isoforms, 22 and 38 kDa - so why is it showing up at the same spot as STRA8?
STRA8 is 37 kDa, so why running so high? This series of western blots (which are not "uncropped", as the title suggests, is a big concern to this reviewer.
**Response:** This is a good question. The predicted size of ACTB is indeed 42 kDa. The difference between original Fig. S9Aan B is due to the different running time and the concentration of the two gels. The band difference in band pattern may be caused by the different materials, one from the P9 testes, the other from the adult ones.
According to the paper by Zhang *et al.*, “ENSEMBL predicts DMRT6 to have two potential protein isoforms of about 22 kDa and 38 kDa. We detected just one strongly expressed protein of about 47 kDa that was specific to wild-type testes (supplementary material Fig. S2C)” (Zhang et al., 2014). The predicted size of STRA8 is 45 kDa but not 37 kDa (https://www.uniprot.org/uniprot/P70278). Therefore, the signals of STRA8 and DMRT6 are close to each. The signals of these proteins were detected by repeatedly stripping and reprobing of the same blot, therefore, the signals between these two proteins are almost indistinguishable. The Ethics Team of the journal has asked us to provide the original TIFF files of these two proteins for investigation and they informed us “These original blots are fine and are different, but they do have similar shapes. I think that it would be good to add a brief sentence to the legend or the Materials and Methods stating that the blots have been stripped and reprobed just to make this clear and to ensure there are no issues after publication, when they would be more difficult to resolve”.

Reviewer 3 Advance Summary and Potential Significance to Field:
This is an interesting and insightful work which mainly focused on the regulation network on meiotic initiation. The authors showed that miR-202, a member of the let- 7 family, prevents precocious spermatogonial differentiation and meiotic initiation in spermatogenesis. Knockout of miR-202 will lead to the reduction of undifferentiated spermatogonial pool and ultimately causing agametic seminiferous tubules through the earlier spermatogonial differentiation and meiotic initiation. While the molecular assays explored the working mechanism of miR-202 in spermatogenesis, which showed that miR-202 directly targets Dmrt6 mRNA and restricts the expression window of DMRT6 in SGdiff, to coordinate an orderly transition from the mitosis to the meiosis. The in vivo and in vitro results could well support the conclusion.

Reviewer 3 Comments for the Author:
there are several minor issues need to be addressed.
1、How is the expression pattern of miR-202 in testis, does it localize in Sertoli cell and affect Sertoli cell functions?
**Response:** The expression of miR-202 was analyzed in our previous report (Chen et al., 2017) and it is indeed expressed in Sertoli cells. In our preprint (https://doi.org/10.1101/2021.04.14.439735), Sertoli cell number was not changed in *miR-202* KO mice. In the revised manuscript, we added transplantation results to show that the colony number of transplanted SSCs in the testes of the KO recipient mice is not significantly different from that of the WT mice, indicating the spermatogenic niche of the KO mice is not disrupted by the KO of *miR-202* genes (Fig. 1E).

2、the in vitro experiments showed the increased apoptosis in ko group, how is going in vivo ?
**Response:** We have performed the apoptosis analysis in vivo by TUNEL assay and found no significant increase of apoptotic cells in KO mice (Fig. S8B). The description has been added to the Result section (lines 243-247). The increased number of apoptotic cells in KO testes *in vitro* may be a combined result of the KO cells and the non-natural growth condition of the *in vitro* system.

3、the authors showed knockout miR-202 lead to advanced differentiation of SG and exhaustion of SG pool. Will it affect SG proliferation？
**Response:** We have performed the proliferation analysis in vivo by staining for KI67 and found that no significant change in spermatogonia (Fig. S8B). The description has been added to the Result section (lines 243-247).

4、In the discussion, it concludes that knockout of miR-202 phenocopy mutant of DMRT1, NANOS2, MAX and AGO4, does miR-202 also target these genes?
**Response:** As miRNAs generally inhibit translation and degrade mRNAs, we infer that it's impossible for *miR-202* to target these genes functionally. Also, these genes are not predicted to be targets of *miR-202-3p* and *miR-202-5p* (Paraskevopoulou et al., 2013; Agarwal et al., 2015).

5、line 225: there are two "in"
**Response:** We have changed it. Thank you very much.

**Reference**

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Second decision letter

MS ID#: DEVELOP/2021/199799

MS TITLE: MicroRNA-202 prevents precocious spermatogonial differentiation and meiotic initiation during mouse spermatogenesis

AUTHORS: Jian Chen, Chenxu Gao, Xiwen Lin, Yan Ning, Wei He, Chunwei Zheng, Daoqin Zhang, Lin Yan, Binjie Jiang, Yuting Zhao, Md Alim Hossen, and Chunsheng Han

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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. In particular I would direct you to the comments of Referee 1 and ask you to follow their suggestion of including one or two sentences in the Discussion that deal with the potential caveats of the Dicer deletions.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

*Advance summary and potential significance to field*

This is an important advance that describes an miRNA critical for the timing of meiosis and spermiogenesis.

*Comments for the author*

The authors have addressed all of my comments in detail. From line 304 the authors discuss their findings in the context of published Dicer deletions. The point they make are good, but in addition they should add a couple of sentences (or a Supp Dicussion) drawing out the potential caveats with those Dicer deletions. To help them, I describe my view on the state of the Dicer / germline field below:
REVIEWER'S COMMENTS ON EXISTING DICER DELETION MODELS
The Ddx4-Cre mediated Dicer deletion (PMID: 21998645) studied a flox/flox Cre model, not a flox/null Cre model. The latter approach would have afforded superior levels of Dicer deletion. Setting this aside, there is no evidence in that ms that total Dicer levels are depleted. The authors only assay the abundance of Dicer exon 24 (which encodes only part of the critical RNAse III domain - not all of it). Total Dicer transcript and protein abundance are never assessed. Furthermore, expression of only two, not all, miRNAs are assayed.
Studies using TNAP-Cre suffered related problems. For instance, in study PMID: 18320056, Dicer deletion is only achieved in 50% of germ cells. In another TNAP-Cre study (PMID: 18633141), Dicer levels are not assessed at all, and, notably expression of a germ cell specific miRNA is reduced, but not abolished. These previous studies come to different conclusions regarding the function of Dicer which vary from a role early (in spermatogonia), slightly later (in meiosis), or later still (in spermatids). This is almost certainly the result of varying Cre efficiencies. One must remember when studying the germline that non-deletant germ cells can often compensate for the mutant, non-viable ones, muddying primary phenotypes. It is therefore critical that conditional systems work really well. A final, related point is that these Dicer studies are germ cell deletions, whereas the Development submission under review deletes the miRNA-202 in all cells of the testis, i.e. germ cells and somatic cells. It is possible that miRNA-202 is acting non-germ cell autonomously.

Reviewer 2

*Advance summary and potential significance to field*

N/A

*Comments for the author*

The reviewer remains unconvinced that the changes made, based on the huge number of conceptual and technical problems outlined in the first review, have been satisfactorily addressed.

Reviewer 3

*Advance summary and potential significance to field*

This paper reported an interesting phenotype of miRNA-202 knockout mice, and carefully studied the underlying mechanisms. It provided a novel understanding of how are the spermatogonial differentiation and meiotic initiation controlled accurately.

*Comments for the author*

All my concerns have been well addressed, I have no more comments.

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**Second revision**

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
This is an important advance that describes an miRNA critical for the timing of meiosis and spermiogenesis.

Reviewer 1 Comments for the Author:
The authors have addressed all of my comments in detail. From line 304 the authors discuss their findings in the context of published Dicer deletions. The point they make are good, but in addition they should add a couple of sentences (or a Supp Dicussion) drawing out the potential caveats with those Dicer deletions. To help them, I describe my view on the state of the Dicer / germline field below:

REVIEWER'S COMMENTS ON EXISTING DICER DELETION MODELS
The Ddx4-Cre mediated Dicer deletion (PMID: 21998645) studied a flox/flox Cre model, not a flox/null Cre model. The latter approach would have afforded superior levels of Dicer deletion. Setting this aside, there is no evidence in that ms that total Dicer levels are depleted. The authors only assay the abundance of Dicer exon 24 (which encodes only part of the critical RNAse III domain - not all of it). Total Dicer transcript and protein abundance are never assessed. Furthermore, expression of only two, not all, miRNAs are assayed. Studies using TNAP-Cre suffered related problems. For instance, in study PMID: 18320056, Dicer deletion is only achieved in 50% of germ cells. In another TNAP-Cre study (PMID: 18633141), Dicer levels are not assessed at all, and, notably, expression of a germ cell specific miRNA is reduced, but not abolished. These previous studies come to different conclusions regarding the function of Dicer, which vary from a role early (in spermatogonia), slightly later (in meiosis), or later still (in spermatids). This is almost certainly the result of varying Cre efficiencies. One must remember when studying the germline that non-deletant germ cells can often compensate for the mutant, non-viable ones, muddying primary phenotypes. It is therefore critical that conditional systems work really well. A final, related point is that these Dicer studies are germ cell deletions, whereas the Development submission under review deletes the miRNA-202 in all cells of the testis, i.e. germ cells and somatic cells. It is possible that miRNA-202 is acting non- germ cell autonomously.
**Response**: Thank you so much for your kindly help! We have added several lines in the Discussion section based on your view on varying Cre efficiencies during germline knockout of *Dicer* (lines 310-315).

Reviewer 2 Advance Summary and Potential Significance to Field:

N/A

Reviewer 2 Comments for the Author:
The reviewer remains unconvinced that the changes made, based on the huge number of conceptual and technical problems outlined in the first review, have been satisfactorily addressed.
**Response**: We thank the reviewer for the critical review of our work. We had carefully addressed

all the concerns by this reviewer in our last revision by adding more results and detailed explanations. Unfortunately, we do not understand why the reviewer thinks that changes made in last revision have not been satisfactorily addressed. With the suggestion and help of the first reviewer, we have added several lines (lines 310-315) in the Discussion section to address the caveats about the seemingly discrepancy in phonotypes between the total miRNA KO mice represented by *Dicer* KO and our *miR-202* KO mice. Other than this issue, we do not think our work has huge number of conceptual and technical problems, particularly after our revision.

Reviewer 3 Advance Summary and Potential Significance to Field:
This paper reported an interesting phenotype of miRNA-202 knockout mice, and carefully studied the underlying mechanisms. It provided a novel understanding of how are the spermatogonial differentiation and meiotic initiation controlled accurately.

Reviewer 3 Comments for the Author:
All my concerns have been well addressed, I have no more comments.
**Response**: Thank the reviewer for careful evaluation of our work again.

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Third decision letter

MS ID#: DEVELOP/2021/199799

MS TITLE: MicroRNA-202 prevents precocious spermatogonial differentiation and meiotic initiation during mouse spermatogenesis

AUTHORS: Jian Chen, Chenxu Gao, Xiwen Lin, Yan Ning, Wei He, Chunwei Zheng, Daoqin Zhang, Lin Yan, Binjie Jiang, Yuting Zhao, Md Alim Hossen, and Chunsheng Han
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.