Author's response to reviews

Title: MicroRNA-26b inhibits epithelial-mesenchymal transition in hepatocellular carcinoma by targeting USP9X

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Author's response to reviews: see over
Dear Editor-in-Chief,

We are the authors of manuscript MS: 9952607961147132. The title of the manuscript is *MicroRNA-26b inhibits epithelial-mesenchymal transition in hepatocellular carcinoma by targeting USP9X.*

In this study, we aim to investigate the role of miR-26b in modulating epithelial-mesenchymal transition (EMT) in hepatocellular carcinoma (HCC), as well as to identify its underlying mechanism of action.

We thank for reviewer’s comments and We have revised the manuscript as the reviewers recommended. The questions that the reviewers requested also were answered point-by-point. As shown in the followings.

Thanks again!

Yours sincerely

Hongyun Jia
The manuscript appears largely ameliorated compared to the original submission. I would suggest the authors to include in the text also the experiments and figures provided to the referees (as main or supplementary text/figure), such as the measurement of mir-26b levels upon inhibition or overexpression (add to fig.2), luciferase assay with mutated 3’UTR (add to fig.4) etc...

Response: We thanks for the reviewer’s suggestion and reviewer’s point is well taken.

1) My major concern is still the use of a unique control (NC) for oligonucleotides utilized for inhibition and overexpression of miR-26b. Actually, if it is clear that antisense oligonucleotide is single-stranded, it is not obvious that the molecule used for miRNA overexpression is single-stranded as well. Generally, double-stranded miRNA mimics or even precursors are used, for which a single stranded NC would not be appropriate. Please clarify this point and eventually repeat the experiments using the appropriate control for each oligo.

Response: We thank for reviewer’s comments and we are sorry that we did not write this point clearly in last submitted manuscript. There are two different controls were used in this study, micrON™ miRNA Mimic Negative Controls and micrOFF™ miRNA Inhibitor Negative Controls, which were purchased from Guangzhou RiboBio Co.. The micrON™ miRNA Negative Controls Mimics are chemically synthesized double-stranded small RNAs which show the minimal homology to all known miRNAs of miRBase 18.0, and it is a crucial experimental control for miRNA “gain-of-function” studies. And the micrOFF™ miRNA Inhibitor Negtive Controls are chemically synthesized single-stranded RNA molecules which are designed for minimum homology to the miRNA being studied, and thus an indispensable control for miRNA functional studies. The appropriate statement has been added into the Material and methods section in revised manuscript.

2) The other issue, already highlighted in my previous revision, is the calculation of p-values in fig.1. It does not make sense to calculate the p-value of the triplicate PCR for each sample compared to the control. Rather, it would be more informative, even if there are only 3 samples/group, to calculate the p-value of the difference between i) all tumors vs all normal tissues, ii) WHO I vs normal tissues and iii) each WHO vs the others. Eventually, iv) all cell lines together vs all normal tissues.

Response: We thanks for the reviewer’s suggestion. We used a two-tailed Student’s t-test to evaluate the statistical significance of the differences between two groups of
data. A P-value less than 0.05 was considered to be statistically significant. The p-value of the difference between:

i) all tumors vs all normal tissues is 0.0001

ii) WHO I vs normal tissues is 0.001258

iii) WHO II vs normal tissues is 0.00087;
    - WHO III vs normal tissues is 0.000163;
    - WHO IV vs normal tissues is 0.000078;
    - WHO I vs WHO II tissues is 0.351421;
    - WHO II vs WHO III tissues is 0.000618;
    - WHO III vs WHO IV tissues is 0.000118

iv) all cell lines together vs all normal tissues is 0.000085

The data have been incorporated into the revised manuscript (Please see new Figure 1 in revised manuscript).

3) Show in the paper the measurement of USP9X mRNA levels upon miR-26b manipulation

Response: We thanks for the reviewer’s suggestion and reviewer’s point is well taken. We checked the mRNA levels of USP9X after miR-26b manipulation by RT-PCR. There’s no change on the mRNA levels of USP9X after miR-26b manipulation. This result and descriptions have been incorporated into revised manuscript.

4) Figure 5: show levels of USP9X protein as reported in the figure 2 for the referee.

Response: We thanks for the reviewer’s suggestion. As reviewer’s requirement, the protein levels of USP9X and E-cadherin have been incorporated into Figure 5B in revised manuscript.

5) The sentence “the levels of cytokines...” in the discussion is still unclear.

Please check English language.

Response: We are sorry for these grammar and spelling errors. We have made careful editing throughout the entire manuscript and have the revised manuscript corrected again by professional editors before this resubmission.
1. In this manuscript the authors studied miR-26b in a hepatocellular carcinoma context. They should specify why they decided to study this miRNA and they should provide some robust reasons for that.

**Answer:** Because an increasing body of evidence indicates miR-26b is downregulated in several cancers, including hepatocellular carcinoma. But the role of miR-26b in hepatocellular carcinoma tumorigenesis and metastasis is incompletely understood.

**New request:** Please, provide this information in the manuscript.

**Response:** We thank the reviewer for the comment, and we are sorry for not providing this information in last submitted manuscript. The description have been incorporated into the revised manuscript (Page 5 last paragraph).

5. The authors used miRNA mimic and miRNA inhibitor as strategies to modulate miRNA levels. They should report the expression of miR-26b after miRNA modulation because this could help them understand why they obtain better results using miR-26 inhibitor than miRNA mimics (as in the case of wound healing assay), as it would be more logical to expect.

**Answer:** We have measured the miR-26b levels in HCC cells upon overexpression and inhibition as shown in Suppl fig 1.

**New request:** Please, provide this information in the manuscript.

**Response:** We thank for the reviewer’s comments and reviewer’s point is well taken. This result and descriptions have been incorporated into revised manuscript. The expression of miR-26b after miRNA modulation have been shown in rearranged Fig 2A.

6. Why did the authors decide to use Huh7 and HepG2 cell lines? Please provide some explanation.

**Answer:** We used Huh7/Hep3B cell line with medium-level of miR-26b expression for both knockdown and overexpression modifications, by which subsequent functional analyses could be performed comparatively among miR-26b-overexpressing, -silenced, and NC control cells that had the same parental genetic background. Using cell line with medium-level of microRNA expression, as having been employed well widely by numerous previously published studies, has proved reliable and suitable in many cases (1-4).

**New request:** Please provide this information in the manuscript.

**Response:** We thank the reviewer for the comment, and we are sorry for not providing this information in last submitted manuscript. The description have been incorporated into the revised manuscript (Page 12 last paragraph).
7. Figure 2A: The authors should clarify what NC means and why they used an Empty vector as a control.

Answer: The NC is the unrelated miRNA that is the random sequence of miR-26b. This NC can’t connect with the USP9X 3’UTR.

New request: The authors should describe the meaning of NC in the manuscript where is necessary.

Response: We thank for reviewer’s comments and we are sorry that we did not write this point clearly in last submitted manuscript. The appropriate statement has been added into the Material and methods section in revised manuscript (Page 7 “Manipulation of miR-26b expression levels” section).

8. Figure 2B: Why did the authors show the amount of USP9X using Western Blotting in this figure? Please, move this blot into a more appropriated figure and assess also the mRNA levels of the gene after miRNA modulation.

Answer: We have moved the figure USP9X expression to the Fig 4 as recommendation. We have checked the mRNA levels of USP9X after miR-26b manipulation by RT-PCR. There’s no change on the mRNA levels.

New request: Please, provide mRNA data in the manuscript.

Response: We thanks for the reviewer’s suggestion and reviewer’s point is well taken. We checked the mRNA levels of USP9X after miR-26b manipulation by RT-PCR. There’s no change on the mRNA levels of USP9X after miR-26b manipulation. This result and descriptions have been incorporated into revised manuscript (Page 14 the second paragraph).

9. Figure 2C: The authors should quantify the results obtained using wound healing assay and they should show pictures at 0, 12 and 24 hours after transfection.

Answer: Here we supplemented the wound healing assay at 0, 12 and 24 hours after transfection.

New request: Please, provide this information in the manuscript.

Response: We thanks for the reviewer’s suggestion and reviewer’s point is well taken. The pictures have been incorporated into the revised manuscript (Please see new Figure 2D in revised manuscript).

11. Figure 3B: the authors should also carry out an E-cadherin staining to assess the capability of this miRNA to increase the amount of this protein in a 3D context.

Answer: We have supplemented the E-cadherin staining as recommended.
New request: Please, provide this information in the manuscript.

Response: We thanks for the reviewer’s suggestion and reviewer’s point is well taken. The E-cadherin staining have been incorporated into the revised manuscript (Please see supplemented Figure 1 in revised manuscript).

12. Figure 4C: The authors showed an immunofluorescence staining for Smad4 to prove the effect of miR-26b on USP9X. They should perform immunofluorescence also using USP9X staining. They should also carry out a western blotting checking the protein levels of USP9X and Smad4 after miRNA modulation. Answer: We have examined the USP9X expression by western blotting (Fig 4B). Because of the time the immunofluorescence staining we didn’t finish this time. And we will continue the examination in the following research.

New request: Please, provide the protein amount of USP9X in the manuscript and perform Immunofluorescence experiment.

Response: We thanks for the reviewer’s suggestion and reviewer’s point is well taken. The datas have been incorporated into the revised manuscript (Please see Figure 2B in revised manuscript).

13. Furthermore, the authors described in the manuscript the biological relevance of USP9X in the axis TGF-β pathway/Smad4. They should assess the effect of USP9X on ubiquitination of Smad4 after miRNA modulation. Answer: we have assessed the effect of USP9X on ubiquitination of Smad4 after miR-26b modulation as shown in Suppl Fig 6. The authors did not provide any effect of USP9X on ubiquitination of Smad4 after miRNA modulation. They should provide robust information about this critical aspect of their manuscript.

Response: We thank the review for pointing out the error, and we apologize for not describing it clearly in the in last submitted manuscript. We have assessed the effect of USP9X on ubiquitination of Smad4 after miR-26b modulation as shown in Supplemental Figure 4.

To better understand the TGF-β pathway/Smad4 axis presented in current study, we transfected miR-26b into the HCC cancer cells. As shown in Supplemental Figure 4, ectopic expression of miR-26b increased the ubiquitination levels of Smad4( Ub-Smad4 is a 75-KDa band reactive to the anti-Smad4 antibody[1]). The abovementioned results and descriptions have been incorporated into revised manuscript.

15. The authors should employ a siRNA for knocking-down USP9X and check if this could phenocopy the effects of miR-26b overexpression.
Answer: Knocking-down USP9X in Huh7 cells, we can found the similar effects of miR-26b overexpression, as shown in Suppl Fig 8.

New request: Please, provide this information in the manuscript. Although the authors performed one experiment using siRNA, they did not verify whether this siRNA can really phenocopy all the miR-26b-mediated effects on the epithelial to mesenchymal transition (For instance, they should assess the effect of siRNA on EMT markers). They should carry out these crucial experiments.

Response: The reviewer’s point is appreciated, and we are sorry for not providing this information in last submitted manuscript. As reviewer suggested, we re-introduced the USP9X siRNA into the miR-26b inhibited HCC cancer cells. As shown in Supplemental Figure 5, ectopic expression of USP9X siRNA increased the expression levels of E-cadherin and decreased the expression levels of vimentin. The abovementioned results and descriptions have been incorporated into revised manuscript.

16. The authors speculated about the link between miR-26b and EMT. They also reported that SNAIL is linked to TGF-β signaling. As they reported an increase of E-cadherin due to miR-26b overexpression, they should assess the mRNA levels of SNAIL after miRNA modulation and after using siRNA for USP9X. In this case, they can gain insight into the mechanisms underlying the link between miR-26b and EMT.

Answer: We examined the Snai1 mRNA after miR-26b overexpression and USP9X silencing as shown in Suppl Fig 9.

New request: Please, provide this information in the manuscript.

Response: We thanks for the reviewer’s suggestion and reviewer’s point is well taken. The description have been incorporated into the revised manuscript( Page 15, the first paragraph), shown in Supplement Figure 2.

17. In the paragraph “USP9X plays an important role in miR-26b suppressed invasiveness of HCC cells” the authors should improve the number of healthy tissues for the estimation of linear regression.

Answer: we have added more tissues for regression analyse.

New request: Please, provide this information in the manuscript.

Response: We thanks for the reviewer’s suggestion and reviewer’s point is well taken. The data have been incorporated into the revised manuscript (Please see new Figure 5B in revised manuscript).

18. Methods: what are the final concentrations of miR-26b mimics, negative control and miR-26b inhibitor? Please add this information. Did the authors use the same
control for miR-26b mimics and inhibitor? In this case, the authors should repeat all the experiments where they used these molecules using appropriate controls for each of them.

Answer: The final concentrations of transfection is 20 nM. miR-26b inhibitor was the chemosynthesis and transfected by lip2000, which can specific recognize the target miRNA, inhibiting the miRNA effects and improve the effector protein expression. We use this to do the loss-of-function research.

New request: Please, provide this information in the manuscript.

**Response:** We thank for reviewer’s comments and we are sorry that we did not write this point clearly in the originally submitted manuscript. The appropriate statement has been added into the Material and methods section in revised manuscript (Page 7, the “Manipulation of miR-26b expression levels” section).

20. Please provide more information about techniques that are used in the manuscript.

For instance, what kind of microscopy has been used.

Answer: The microscopy we have used as:
Zeiss Axio Imager A1——Zeiss, Oberkochen, Germany
Zeiss Axiovert 40c——Zeiss, Oberkochen, Germany
Axiovert 200——Zeiss, Oberkochen, Germany

New request: Please, provide this information in the manuscript.

**Response:** We thank for reviewer’s comments and we are sorry that we did not write this point clearly in the originally submitted manuscript. The appropriate statement has been added into the Material and methods section in revised manuscript.

- Even if the authors said in their report that they corrected the mistakes, there are still some discrepancies about magnification between what the author have written in the figure legends/materials and methods and figures. This aspect is critical because this mistake is repeated. Could you please provide in the supplementary figures or just in your new report others pictures about this experiment using different magnification?

**Response:** We thanks for the reviewer’s suggestion and reviewer’s point is well taken.