Author’s response to reviews

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

Authors:

Gang Shen (gs1975@163.com)
Ye Lin (linye322@hotmail.com)
Xuewei Yang (yangxw0908@163.com)
Jing Zhang (fejr@foxmail.com)
Zhe Xu (Drzhexu@163.com)
Hongyun Jia (hongyun_jia@163.com)

Version:3 Date: 16 March 2014

Author’s response to reviews: see over
Reviewer's report

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

Version: 2 Date: 13 January 2014

Reviewer: Paolo Gandellini

Reviewer's report:

In the present work, the authors show that miR-26b, down-modulated in HCC, may affect tumor invasion by repressing USP9X. The data are quite well presented, however some major revisions have to be made before the manuscript may be suitable for publication.

Major compulsory revisions:

1) Figure 1: please explain how p-values have been calculated for single tumors and compared to what. I would calculate p-values not for single tumors but for groups: normal vs WHOI etc... and also between different tumor grades.

Answer: The expression of miR-26b in every tumors was measured by Real-time PCR and repeated three times. The average primitive results was compared with the normal tissues by student t test. We mapping the relative results as the normal tissue as 1.

2) Which type of molecule has been used as miR-26b mimics? Please specify.

Answer: The miR-26b mimics was the chemosynthesis. We purchased these mimics from from RiboBio company, Guangzhou, Guangdong, China. The mimics was transfected into HCC cells by lip2000.

3) Which type of molecule has been used as miR-26b inhibitor? It seems that the same negative control molecule has been used for mimics and inhibitor. Please specify.

Answer: miR-26b inhibitor was the chemosynthesis and transfected by lip2000, which can specific recogenize the target miRNA, inhibiting the miRNA effects and improve the effector protein expression. We use this to do the loss-of-function
4) Please measure (by realtime PCR) miR-26b levels in HCC cells upon overexpression and inhibition. In general the authors observe effects (for example on migration) with higher magnitude upon inhibition rather than overexpression of miR-26b. This may imply that the used cell lines have remarkably high endogenous miRNA levels, which would be in contrast with the down-regulation of the miRNA in tumors. Please discuss.

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

Suppl Fig 1 The expression of miR-26b upon overexpression(A) and inhibition(B) by real-time PCR

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).

**References**


5) Please check the effect of miR-26b manipulations also on the mRNA levels of USP9X. 

Answer: We have checked the mRNA levels of USP9X after miR-26b manipulation by RT-PCR. There’s no change on the mRNA levels.

6) Page 12, first paragraph lacks conclusions. 

Answer: We have added the conclusions: “Taken together these results indicate that miR-26b is downregulated in HCC, and correlates with the progression of human hepatocellular carcinoma”. The correction also was done in the manuscript.

7) Page 13: please provide a more detailed description on how the sites for miR-26b in the 3’UTR of USP9X have been identified and specify that the sites are two. 

Answer: The first position is on the 1132-1139 and the second position is on the 1317-1323 of USP9X 3’UTR.

8) Page 14: last paragraph. The authors claim that they used USP9X and USP9X-3’UTR vectors to overexpress USP9X. However, the name USP9X-3’UTR had been previously used to describe the reporter GFP or luciferase vector where only the 3’UTR of USP9X had been cloned. Please provide a more detailed description on how “all” the vectors used in the study have been prepared. Please describe GFP-tubulin vector. 

Answer: In the figure 4, we subcloned the USP9X 3’UTR fragment into PEGFP-C1
and pGL3 dual luciferase reporter vectors. While the GFP-tubulin vector is we cloned the GFP at the N-terminus on Tubulin as a tag.

Page 14: last paragraph: We co-transfected miR-26b mimics and USP9X without 3’UTR into Huh7 and Hep3B cells. And also co-transfected miR-26b mimics and USP9X with 3’UTR into Huh7 and Hep3B cells. To investigate the effects of miR-26b is by inhibiting the expression of USP9X.

We have changed this paragraph as:

To further investigate the role of increased USP9X expression in cells with suppressed miR-26b levels and increased invasiveness, the effects of **USP9X without 3’UTR** and **USP9X with 3’UTR** were examined in cells transfected with miR-26b mimics. The migration assay showed that co-transfection of miR-26b and USP9X without 3’UTR significantly improved the migration rate of both Huh7 and Hep3B cells. However, the combination of **USP9X with 3’UTR** and miR-26b had no obvious effect on migration, compared to cells transfected with miR-26b and vector (Figure 5A).

9) Fig. 5A: miR-26b overexpression may simultaneously affect the expression of endogenous and vector-encoded USP9X. Please discuss this point and add quantification of USP9X expression levels in all combinations. Please assess the expression of EMT markers.

Answer: We have examined the USP9X expression levels in all combinations by western blotting as shown in Suppl Fig 2.

**Suppl Fig 2**  the influence of miR-26b on the expression of E-cadherin
10) Fig 5B: too few samples for regression analyses. The trend is given by the 2 normal tissues. Please repeat the analysis with more samples. Eventually plot E-cadherin and USP9X mRNA expression levels found in samples from fig.1. **Answer:** we have added more tissues for regression analyse. As shown in Suppl Fig 10. We also have substiuted the Fig 5B with this figure.

**Suppl Fig 10** The relative expression level of miR-26b (upper), the corresponding expression level of USP9X, E-cadherin and β-actin are shown in the same samples (left panel). Linear regression analyses of miR-26b expression with USP9X and E-cadherin are also presented (right panel).

11) Check the order of citation of references throughout the paper. Minor essential revisions:

1) Fig 2B anticipates the effect on USP9X protein levels, which should be moved to Fig.4. **Answer:** We have modified as the suggestion.

2) Page 10, last paragraph: pGL3-SNAI1-3’UTR is cited in the methods. Please correct. **Answer:** We have corrected as the pGL3-USP9X-3’UTR.

3) Page 11, last paragraph: nine samples are cited in the text but twelve are shown in the figure (Fig. 1) **Answer:** We have corrected as show in the figure, we have tested twelve archived
clinical HCC samples.

3) Page 17: the authors claim that suppression of luciferase activity is dependent on miR-26b binding sequence. Though it is clear that the used fragment of USP9X 3’UTR is responsive to manipulation of miR-26b levels, direct proof would come from luciferase experiments carried out using 3’-UTR with mutated miR-26b sites. Please perform such experiments, or alternatively rephrase/discuss. At least show that modulation of an unrelated miRNA (not having its target sequence in the 3’UTR fragment cloned in the vector) does not affect luciferase activity.

**Answer:** In this paper we have used NC as control. The NC is the unrelated miRNA that is some random sequence. This NC can’t connect with the USP9X 3’UTR.

5) Discussion, last paragraph: a role of mir-26b as prognostic factor has been proposed but this completely lacks experimental validation. The data shown by the authors do not provide any evidence of this. Please perform the analyses, or, alternatively, rephrase.

**Answer:** We have rephrased the last paragraph. As shown: **Our data suggests that miR-26b may play some roles in the EMT of HCC cells, although future studies are required to confirm this.**

6) Check punctuation and spelling. Avoid repetitions.

**Answer:** We have checked the punctuation and spellings of this paper.

7) Discussion: Line 9 and line 10 (after citation of ref. 28) are not logically linked. Please rephrase.

**Answer:** We have rephrased the last paragraph. As shown: **The levels of cytokines driving EMT are known to be controlled by miRNA [33]. While miR-26a and miR-26b have been reported can regulate the NF-κB, TGF-β signal pathways[34]. And miR-26b was downregulated in hepatic tumors, as compared to paired noncancerous tissue[35].**
We have checked and updated the references as recommendation.
Reviewer's report 2
Title: MicroRNA-26b inhibits epithelial-mesenchymal transition in hepatocellular carcinoma by targeting USP9X
Version: 2 Date: 14 January 2014
Reviewer: Nicola Fenderico

Reviewer's report:
Overall, the manuscript by Shen X et al. is sufficiently original. However, the manuscript needs important and essential changes in order to make it more complete and grounded. For these reasons, a major revision is required.

Major Compulsory Revisions
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.

2. They should provide the average miR-26b expression levels for each different grade.
Answer: Because there were only three samples for each grade, we didn’t provide the average to represent the expression levels. And we will continue to collect the samples to verify the conclusion of this paper.

3. Moreover, at page 11, line 17 the authors mentioned 9 clinical samples but they showed 15. Please, correct these mistakes.
Answer: We have corrected these mistakes. Show as 3 normal liver tissues and 12 archived clinical HCC samples.

4. This paragraph misses a conclusion because the last sentence is meaningless.
Answer: We have rephrased the last sentence as: *Taken together these results indicate that miR-26b may be an tumor inhibitor in the progression of human hepatocellular carcinoma.*

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.

![Suppl Fig 1](image)

**Suppl Fig 1** The expression of miR-26b upon overexpression(A) and inhibition(B) by real-time PCR

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).
References


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.

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.

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. As shown in Suppl Fig 3.
Suppl Fig 3  The wound healing assay at 0, 12 and 24 hours after transfection.

10. Moreover, in material and methods they declared to use 20X magnification and in the legend of this figure 200X magnification. Please, correct this mistake.

**Answer:** We have corrected the magnification in figure as 20X.

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. As shown in Suppl Fig 4.
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.

The Smad4 expression after miRNA manuscript as shown in Suppl Fig 5:
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.

Figure 4D: The authors reported a luciferase assay in order to demonstrate the direct binding of miR-26b in the 3’UTR of USP9X. Because they found 2 different miR-26b binding sites in the 3’UTR of USP9X, they should mutate miR-26b binding sites (one by one and together) and assess if this is able to induce an escape of miRNA-mediated inhibition.

Answer: We have supplemented this experiment and the results shown in Suppl Fig 7:
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.

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

![Graph showing relative SNA1 mRNA expression with NC, miR-26b, and siUSP9X treatments for Huh7 and Hep3B cells.](image)

**Suppl Fig 9** The Snai1 mRNAs expression after miR-26b overexpression and USP9X silencing

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. As shown in Suppl Fig 10. We also have substituted the Fig 5B with this figure.

![Suppl Fig 10 showing relative expression level of miR-26b and USP9X, E-cadherin, and β-actin](image)

**Suppl Fig 10** The relative expression level of miR-26b (upper), the corresponding expression level of USP9X, E-cadherin and β-actin are shown in the same samples (left panel). Linear regression analyses of miR-26b expression with USP9X and E-cadherin are also presented (right panel).
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.

19. Moreover, they should provide the appropriate company for all reagents that they used for this manuscript (For instance, antibodies).

**Answer:** We have corrected as the recommendation.

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 —— Zeis, Oberkochen, Germany

21. Why the authors reported the use of microarray if this technique is not cited in the manuscript?

**Answer:** There were no microarray data in this paper. We have deleted the microarray in manuscript.

22. The manuscript needs English revision

**Answer:** We have asked for the English revision by those whose mother tongue is English.
23. Some mistakes in the form (For instance: pag. 4, line 3, lack of space; pag.4, line 5, full stop instead of comma; Figure legend 5, title.)

Answer: We have corrected these mistakes.

24. In the figure legends the authors should write the letter of corresponding figure panel before the explanation of each figure.

Answer: We have corrected as recommendation.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Needs some language corrections before being published

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests