Reviewer's report

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

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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

• 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.
• They should provide the average miR-26b expression levels for each different grade.

Moreover, at page 11, line 17 the authors mentioned 9 clinical samples but they showed 15. Please, correct these mistakes.

This paragraph misses a conclusion because the last sentence is meaningless.
• 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.
• Why did the authors decide to use Huh7 and HepB cell lines? Please provide some explanation.
• Figure 2A: The authors should clarify what NC means and why they used an empty vector as a control.
• 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.
• 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.

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

- 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.

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.

- 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.

- The authors should employ a siRNA for knocking-down USP9X and check if this could phenocopy the effects of miR-26b overexpression.

- 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.

- 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.

- 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.

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

Please provide more information about techniques that are used in the manuscript. For instance, what kind of microscopy has been used.

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

- The manuscript needs English revision

- 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.)

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