Author's response to reviews

Title: MiR-125b promotes proliferation and migration of type II endometrial carcinoma cells through targeting TP53INP1 tumor suppressor in vitro and in vivo

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

Thank you very much for your kindly consideration! In response to the suggestions and comments raised by the editor and referees we have made a major revision in the manuscript. Furthermore, all changes of revision have been marked by highlight in blue in our corrected version. Our point-to-point responses are listed below:

Burton Yang Reviewer’s report:

Major comments:

1/ As indicated the title ‘miR-125b promotes proliferation and migration of type II endometrial carcinoma cells through targeting TP53INP1 tumor suppressor in vitro and in vivo’, the authors, however, failed to provide sufficient evidence to reach this conclusion. The authors only demonstrated that TP53INP1 is the target of miR-125b. There is no data to show that the role of miR-125b on cell proliferation and migration occurred through TP53INP1. To draw this conclusion, the authors need to use siRNA against to confirm a similar result could be derived and over expression of TP53INP1 can rescue the effects of miR-125b. It is noted that the authors have performed a large amount of work. These comments are trying to help the authors to improve their manuscript.

Answer: Thank you for your suggestions. To inhibit expression of endogenous TP53INP1, we designed and prepared HPLC-purified TP53INP1 siRNA (sense: 5’-TCAGCCTCTGGAACAT-3’; anti-sense: 5’-ATGTTCCAGGGCTGA-3’) and, A scrambled siRNA with no homology to
any known human mRNA was used as negative control (sense: 5'-CTGTTAAAAATCCAGG-3'; anti-sense: 5'-CCTGGATTAAAAACAG-3') according to the sequence of the TP53INP1 gene. siRNA oligonucleotide duplexes were synthesized by Genephama Biotech (Shanghai, China). To address whether the above-observed phenotype is indeed due to the suppression of TP53INP1 and not from the targeting of other cellular genes by miR-125b, a rescue experiment was performed. We transfected AN3CA cells with miR-125bi plus siTP53INP1 (TP53INP1 siRNA) or with the same inhibitors plus siTP53INP1 control. At 48h posttransfection, western blot revealed that the expression level of TP53INP1 protein in cells cotransfected with miR-125bi plus siTP53INP1 was significantly lower than that in cells cotransfected with miR-125bi plus siTP53INP1 control (Figure 6A~B). Indeed, AN3CA cells with stably repressed TP53INP1 were enhanced in both proliferation and migration ability (Figure 6C, P<0.05 and Figure 6D, P<0.01). These results imply that repressing TP53INP1 expression could significantly attenuate the inhibitory effect of miR-125bi on cell proliferation and migration, suggesting that the miR-125b promoted the proliferation and migration of AN3CA cells through targeting TP53INP1 signal pathway.
Figure 6. A~B. The expression level of TP53INP1 protein was detected by western blot at 48h posttransfection and normalized to that of β-actin. The expression level of TP53INP1 protein was significantly lower in cells cotransfected with miR-125bi plus siTP53INP1 as compared to the cells cotransfected with miR-125bi plus siTP53INP1 control (*P<0.01). C. Cell proliferation was evaluated by CCK8 analysis. The proliferative capacity of AN3CA cells cotransfected with miR-125bi plus siTP53INP1 was significantly higher than that of cells cotransfected with miR-125bi plus siTP53INP1 control (*P<0.05). D. Cell migration of AN3CA was determined by transwell migration analysis. The number of migrated cells of AN3CA cells cotransfected with miR-125bi plus siTP53INP1 was increased about 2.5 fold as compared that of cells cotransfected with miR-125bi plus siTP53INP1 control (*P<0.01).

2/ On the wound healing assay and tumor formation assay, ishikawa cells transfected with miR-125bm should be compared with AN3CA-NC cell, as it might be worthy to see the difference between the miR-125b-overexpressed ishikawa cells with high endogenous miR-125b AN3CA cells. **Answer:** Thank you for your suggestions. Ishikawa cells and AN3CA cells are both endometrial carcinoma cells, however, Ishikawa cells belong to gland epithelial cells and AN3CA cells belong to epithelial cells like epithelial ovarian cancer cells. Cell morphology of them can not change each other by transfection of miR-125bm or miR-125bi. Besides, volume of the equal number cells is different. Therefore, On the wound healing assay and tumor formation assay, It is not suitable for comparison between the miR-125b overexpressed ishikawa cells and high endogenous miR-125b AN3CA cells.

3/ In luciferase assays, a scrambled sequence is needed as a negative control. **Answer:** Thank you for your suggestions. MiR-125bm sequence (Sense 5’-UCCUGAGACCCUAACUUGUGA-3’, Anti-sense 5’-ACAAGUUAGGGUCUCAGUGAUU-3’) is identical with miR-125b,
MiR-125bm NC sequence (Sense 5'-UUCUCCGAACGUGUCACGUTT-3', Anti-sense 5'-ACGUGACACGUUCGGAGAATT-3') is equivalent to scrambled sequence and used as a negative control.

4/ In tumor formation assay that shown in Fig. 6B, injection of non-treated cells and vector cells (2 and 3) should be performed onto the same mouse in order to compare if there is any effect in vector transfection of cells or not.

Answer: Thank you for your suggestions. One study group in our laboratory did the same work as your description using ishikawa cells transfected with the same empty plasmid and non-treated ishikawa cells for other miRNAs research. They found no statistical difference between the two groups after injection into the bilateral hind leg subcutaneous tissue of four-week old female BALB/C athymic nude mice. However, the results of the research was not published. Therefore, we did not need to repeat the same work.

5/ Fig 6E, the Ki67 staining appears to have much non-specific staining. A good staining should produce clear-cut result, rather than a gradient from strong to weak staining.

Answer: Thank you for your suggestions. The focal length of the microscope was not adjusted properly, so photographs were not clear. Partial photographs of ki67 staining were taken again as follow (Figure 7).
Figure 7 Tumorigenicity assay in nude mice. A. Tumor growth curve in nude mice. After tumor cells were injected subcutaneously into the bilateral hind leg of nude mice, the short and long diameters of the tumors were measured weekly and tumor volumes (cm^3) were calculated. B. the nude mice with tumor formations. ① indicates the nude mouse injected with Ishikawa cells transfected with miR-125b plasmid. ② indicates the nude mouse injected with Ishikawa cells transfected with control vector. ③ indicates the nude mouse injected with Ishikawa cells with no treatment. C. Photograph of tumors derived from miR-125b plasmid, control vector, or untransfected Ishikawa cells in nude mice. D. Weights of tumors. *P<0.01 as compared with either no transfection group or control vector group. E. Representative HE staining histopathologic image of tumor tissues in mice. (upper panel). Ki67 expression of tumors was detected by immunohistochemical techniques (lower panel).

Minor points:
1/ On page 11, line 261, was ‘aslo’ performed…

*Answer:* Thank you for your suggestions. We have changed the original manuscript into the corrected description.

2/ On page 17, line 328, ‘using archival archival primary ECs tissue samples’….

*Answer:* Thank you for your suggestions. We have changed the original manuscript into the corrected description.

3/ On page 18, line 347, ‘we nest asked’….

*Answer:* Thank you for your suggestions. We have changed the original manuscript into the corrected description.
Nehad Alajez Reviewer's report:

Major Compulsory Revisions

1. Is the phenotype observed in the experiments in figures 2,3,4, and 6 mediated by TP53INP1? The authors can perform either rescue experiments (to reverse the phenotype observed when overexpressing miR-125b) or at least to knockdown the expression of TP53INP1 in EC I cells to see if this would mimic the effects of miR-125b overexpression.

Answer: Thank you for your suggestions. To inhibit expression of endogenous TP53INP1, we designed and prepared HPLC-purified TP53INP1 siRNA (sense: 5'-TCAGCCTCTGGAACAT-3'; anti-sense: 5'-ATGTTCCAGAGGCTGA-3') and, a scrambled siRNA with no homology to any known human mRNA was used as negative control (sense: 5'-CTGTTAAAAATCCAGG-3'; anti-sense: 5'-CCTGGATTTTTAACAG-3') according to the sequence of the TP53INP1 gene. siRNA oligonucleotide duplexes were synthesized by GenePhama Biotech(Shanghai, China). To address whether the above-observed phenotype is indeed due to the suppression of TP53INP1 and not from the targeting of other cellular genes by miR-125b, a rescue experiment was performed. We transfected AN3CA cells with miR-125bi plus siTP53INP1 (TP53INP1 siRNA) or with the same inhibitors plus siTP53INP1 control. At 48h posttransfection, western blot revealed that the expression level of TP53INP1 protein in cells cotransfected with miR-125bi plus siTP53INP1 was significantly lower than that in cells cotransfected with miR-125bi plus siTP53INP1 control (Figure 6A~B). Indeed, AN3CA cells with stably repressed TP53INP1 were enhanced in both proliferation and migration ability (Figure 6C, P<0.05 and Figure 6D, P<0.01). These results imply that repressing TP53INP1 expression could significantly attenuate the inhibitory effect of miR-125bi on cell proliferation and migration,
suggesting that the miR-125b promoted the proliferation and migration of AN3CA cells through targeting TP53INP1 signal pathway.

Figure 6 A~B. The expression level of TP53INP1 protein was detected by western blot at 48h posttransfection and normalized to that of β-actin. The expression level of TP53INP1 protein was significantly lower in cells cotransfected with miR-125bi plus siTP53INP1 as compared to the cells cotransfected with miR-125bi plus siTP53INP1 control (*P<0.01). C. Cell proliferation was evaluated by CCK8 analysis. The proliferative capacity of AN3CA cells cotransfected with miR-125bi plus siTP53INP1 was significantly higher than that of cells cotransfected with miR-125bi plus siTP53INP1 control (*P<0.05). D. Cell migration of AN3CA was determined by transwell migration analysis. The number of migrated cells of AN3CA cells cotransfected with miR-125bi plus siTP53INP1 was increased about 2.5 fold as compared that of cells cotransfected with miR-125bi plus siTP53INP1 control (*P<0.01).

2. What is the expression level of TP53INP1 in EC I vs EC II primary tissues? Is there inverse relationship between the expression of TP53INP1 and miR-125b?
Answer: Thank you for your suggestions. In our preresearches, we detected endogenous TP53INP1 expression on the archival paraffin-embedded endometrial carcinoma specimens of between type I (endometrioid) and type II (papillary serous) patients (each 10 cases) by Immunohistochemistry and found that TP53INP1 was expressed in 8/10 type I EC and 1/10 type II EC (Figure 8A), meanwhile, we detected endogenous miR-125b expression by qRT-PCR(Figure 8B) and found that the expression level of miR-125b was inversely to endogenous TP53INP1 expression in EC I vs. EC II primary tissues. However, the number of the samples are too small to reach a solid conclusion. We did not present the results in this paper, because this research was supported by another grant.

Figure 8. (A) Endogenous TP53INP1 expression was detected on the type I (endometrioid) and type II (papillary serous) patients (each 10 cases) archival paraffin-embedded endometrial carcinoma specimens by Immunohistochemistry. TP53INP1 was expressed in 8/10 type I EC and 1/10 type II EC. (B) qRT-PCR was performed to validate the expression of miR-125b in type I and type II (each 10 cases) endometrial carcinoma samples. We found that endogenous miR-125b expression was the most significantly up-regulated in type II endometrial carcinoma samples (9/10) compared with type I.
3. The authors referred to their previous microRNA microarray in type I and type II EC cells. Are those data published? In not, is it possible to include at least themiR-125b expression data to confirm the clinical relevance of the findings in primary cancer specimens.

**Answer:** Thank you for your suggestions. These data of the previous profiles of miRNAs (Figure 9) was not published. We can not present the results in this paper, because the profiles of miRNAs was performed by Xiaoyue Chen and will be presented in another paper. We selected significantly up-regulated miRNAs (n=6, miR-125b, miR-196b, miR-625, miR-196a*, miR-29a, and miR-140-5P) and validated their expression in type I and type II (each 10 cases) endometrial carcinoma samples by qRT-PCR (Figure 10). We found that endogenous miR-125b expression was the most significantly up-regulated in type II endometrial carcinoma samples compared with type I. Therefore, we further research the functions of miR-125b in endometrial carcinoma.
Figure 9 Profiles of miRNAs in estrogen receptor negative endometrial carcinoma cells (KLE, AN3CA) and estrogen receptor positive endometrial carcinoma cells (RL95-2, ISHIKAWA).
Figure 10 qRT-PCR was performed to validate the expression of miR-125b, miR-196b, miR-625, miR-196a*, miR-29a, and miR-140-5P in type I (endometrioid) and type II (serous papillary) (each 10 cases) endometrial carcinoma samples.

Minor Essential Revisions

1. The authors claim ref#22 as their own work, but this is not true?

Answer: Thank you for your suggestions. For this error, we are very sorry. We cited ref#22 in order to let readers to know why we choose the four cell lines (RL95-2, ishikawa, KLE and AN3CA) as the type I and type II endometrial cancer cells and what is similarities and difference between our choose and ref#22. Unfortunately, it lead to misunderstanding. We have deleted the ref#22.
2. In Fig 2C and 2D, why is the doubling time different even for the untreated cells?

**Answer:** Thank you for your suggestions. AN3CA cell line in Fig2D is metastatic type II endometrial carcinoma cell, however, ishikawa cell line in Fig2C is well differentiated type I endometrial carcinoma cell. AN3CA cells with endogenous highly expressed miR-125b are more malignant than ishikawa cells with endogenous lowly expressed miR-125b. It may be the explanation that untreated AN3CA cells have short doubling time compared with untreated ishikawa cells.