Author's response to reviews

Title: Fibulin-5 inhibits hepatocellular carcinoma cell migration and invasion by down-regulating matrix metalloproteinase-7 expression

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Author's response to reviews: see over
Response to reviewer 1
1) In Figure1-C, the authors should investigate into the statistically significant difference between the highly metastatic HCC cell lines (MHCC97H and HCC-LM3) and the less metastatic cell lines (Hep3B, HepG2, MHCC97L, and SMMC-7721) in the transcriptional level of Fibulin-5.

Fibulin-5 expression in the highly metastatic HCC cell lines, MHCC97H and HCC-LM3, were obviously lower than those in the low metastatic HCC cell lines including HepG2, MHCC97L, SMMC-7721 and Hep3B.

2) In Figure4-C and –D, the authors have shown the inverse expression pattern of Fabulin-5 and MMP-7 in the clinical specimens. The authors should mention whether the heterogeneous expression pattern exists or not. If so, they should evaluate the expression levels in both the invasive area and central area of each tumor tissue, respectively.

We found that in cases of high Fibulin-5 protein expression, there was no detectable MMP-7 protein expression in the same tissue section. In contrast, in the case of low Fibulin-5 protein expression, there was strong MMP-7 protein expression.

3) In Figure5-C and –D, the authors compared the migration and invasion properties between control and double si-RNA-mediated knockdown of Fibulin-5 and MMP-7. The authors should also compare with single si-RNA-mediated knockdown of Fibulin-5 or MMP-7 in the same experiment. Otherwise, they cannot precisely evaluate the importance of MMP-7 as a target molecule of Fabulin-5.

Restoring MMP-7 can abolish the effect of Fibulin-5 overexpression on anti-metastasis in HCC, suggesting that Fibulin-5 functions as a metastasis-suppressor gene by downregulating MMP-7. Further studies are needed to precisely evaluate the importance of MMP-7 as a target molecule of Fibulin-5.

[Minor Essential Points]
1) The authors have mentioned Fibulin-5 as a tumor-promoting factor depending on the tissue/ cellular context. They should also add the following paper as a reference. Fibulin-5 has been reported to bind with Nogo-B and the complex promotes the migration and invasion capacities of HeLa cells by M. Preis, T et al.(doi:10.1016/j.bbrc.2010.06.068).

We have added this paper.

2) In Figure1, the authors should show the expression difference of Fibulin-5 among HCC cell lines by Western-blotting analysis. After all, the transcriptional difference is not necessarily accompanied by the difference in protein level. All cell lines were subjected to WB for Fibulin-5, our data showed the same pattern as mRNA levels.
Response to reviewer 2

1. Materials and Methods section: the reference to the Boyden chamber assay protocol is a review that does not contain relevant information.
   We have changed this reference.

2. Results: Fig 1a – it is not clear how the quantification of the protein expression was performed.
   We have re-organized Fig 1.

3. Results: Fig 1C shows the differences in the mRNA expression level, what about the protein expression? Does it follow the mRNA pattern?
   All cell lines were subjected to WB for Fibulin-5, our data showed the same pattern as mRNA levels.

4. Fig 3A shows up-regulation of FBLN5 in HCC-LM3 after retroviral infection. It presents result of n=6 samples? Experiments? Clones? Retroviral infection suggests that after infection there is no need for cloning because of high efficiency of the process. What was the efficiency of infection in HCC cells? What is meant under n=6?
   The efficiency of infection in HCC cells is above 90%. n = six independent experiments.

5. Fig 3C shows suppression of invasion and motility of HCC cells. Changes in invasion and motility could be a result of changes in apoptosis, proliferation and cell adhesion. Authors claim that there was no effect on proliferation and apoptosis, but do not show the data, It will add to the conclusions if data will be presented. The influence of FBLN5 overexpression on the adhesion of HCC cells should be tested.
   We have added the data for proliferation and apoptosis.

6. Fig 3B and D what is presented on the upper and lower plates?
   We have added the description in Fig3B and D.

7. The conclusion that FBLN5 exerts an anti-metastatic effect on HCC based merely on changes in motility and invasion seems premature.
   We have changed this description.

8. Authors tested expression of MMP7. Why? Other MMPs seem to be regulated by FBLN5 and play role in invasion and metastases, why there were not tested?
   Because of previous study has reported that Fibulin-5 has also been implicated in inhibiting lung cancer metastasis by modulating matrix metalloproteinase7 (MMP7) expression.

9. What is the magnification in Fig 4C panels I and II?
   They are the same as III and IV.

10. Authors demonstrate inverse correlation between FBLN5 and MMP7 expression on protein level. What about MMP7 processing and activity?
    We will check these contents in further study.

11. Does the level of MMP7 expression inverse correlate with the level of FBLN5 expression in the panel of HCC cell lines and the control?
    In Fig 4A and 4B, Fibulin-5 impaired HCC-LM3 cells showed strong expression
of MMP7 protein. Meanwhile, Fibulin-5 expressed MHCC97L cells showed weak expression of MMP7 protein.

12. Fig 4D. What was the expression of MMP7 in the adjacent noncancerous tissues?

Previous reports have shown that MMP7 is overexpressed in the tissues of HCC as compared with that in the adjacent noncancerous tissues (Dai CX et al. BMC Cancer. 2009 Dec 1;9:418. Gao Q et al. Cancer Sci. 2011 Aug;102(8):1522-31.)

13. Discussion: It has been shown earlier that FBLN5 stimulates EMT via MMP activation. Authors should comment on these data.

We have added these comments.