Author's response to reviews

Title: Sphered cell subpopulations with cancer stem cell properties in human hepatoma cell lines

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Author's response to reviews: see over
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Dear Dr. Tim Shipley:

Thank you for your kind letter dated Feb 7th, 2011, requiring a revision of our manuscript (MS: 1591875628468550). We acknowledge the executive editor and the reviewers for their comments and suggestions, which have helped us improve the manuscript. **We revised the manuscript in accordance with the reviewers’ comments and highlighted all significant changes** and carefully proof-read the manuscript to minimize typographical, grammatical, and bibliographical errors. Attached please find the description on our response to each of the points raised by the reviewers and the editors. We hope that the revised manuscript will meet the standards and lead to publication in *BMC Gastroenterology*.

Here we would like to make a statement for changing the author listing on the revised version. Beibei Zhai has been added to the third place, who has contributed to the final results in the revised version of this paper. All authors have agreed to the author listing change.

We deeply appreciate your time on our manuscript, and look forward to hearing from you.

Sincerely yours,

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Response

Dear Reviewers:

We appreciate the reviewers’ constructive comments. Below is a point by point response to the comments.

Reviewer Malcolm Alison:

1. Tantalising evidence was presented for a role of Notch signalling in the maintenance of sphere-forming cells; the paper would be greatly strengthened by functional studies of knock-down of Notch components.

Response:

Thanks for your good suggestion. Blocking Notch pathway was performed by a gamma-secretase inhibitor MRK003. The secondary dissociated PLC/PRF/5 spheroid cells were treated with 10 µM MRK003 or DMSO control for 7 days. The inactivation of Notch1 and down-regulations of DTX1 and Ep300 were confirmed by Western blotting. The sphere-forming ability of MRK-treated groups was significantly inhibited in comparison to DMSO-treated controls. The results indicated that the CSL-independent Notch signaling pathway might play an important role in liver CSCs, and MRK003 could partly eliminate the stem-like cells (Also shown on Page 12 and Fig. 5 in revised version).


Response:

Thank you. We have made the expression unified as “spherated cells”. The term is more common than others.

3. Fig. 2B: cell survival rate incorrect (a rate has units of time)

Response:

Thanks. The figure has been corrected, and the cell survival data in Y axis is in
“percentages”.

4. Page 10 ? in text, refer to Fig. 4A. Also, the Figure does not support the statement that most cells express OV6.

**Response:**

The OV6 protein is a membrane antigen, and located on cell surfaces which could be observed as red fluorescent labeled circularity in most of cells in figure 4A. The exceptional red spots on the picture are non-specific staining, maybe due to the impurities of the slide.

**Reviewer Nicholas Barker:**

1. The authors report that different cell-lines harbor sub-populations with sphere-forming capabilities in-vitro. However, there is no attempt to compare the efficacies of non-tumorigenic versus highly tumorigenic (eg, HepG2 vs MHCC97H) cell-lines, or Hep+ versus Hep- in this assay. Such information is crucial for drawing generally applicable conclusions.

**Response:**

Thank you for pointing out the important issue. In our preliminary experiment, we’ve compared the tumorigenic efficacies of the three cell lines HepG2, PLC/PRF/5 and MHCC97H in nude mice (malignant grade HepG2<PLC/PRF/5< MHCC97H). The parental cells and spheree cells were injected into nude mice subcutaneously at different cell concentrations, and sacrificed at day 30th. As showed in figure S1, the HepG2 parental cells at $10^6$ cells/mouse could not form a visible xenografts (0/5), while the same amount of PLC/PRF/5 or MHCC97H parental cells could (5/5), and the more malignant MHCC97H cells formed greater volume of tumors. In comparison, the HepG2 spherical cells at $10^6$ cells/mouse and the PLC/PRF/5 or MHCC97H spherical cells at $10^4$ cells/mouse could form xenograft tumors in the same period of 30 days and the volume of tumors was positively correlated with malignant grade of the
cell lines. The results suggested that the tumorigenic efficacies of spheroid cancer cells were enhanced compared with the parental cells (Also shown on Page 10-11, Page 14 and Fig. S1 in the revised version).

2. Related to the above point, it is also important to directly compare the sphere-forming and in-vivo tumorigenic efficacies of at least one other cell-line in addition to PLC/PRF/5 and relate this to CSC activity/frequency.

Response:

As showed above, we compared the tumorigenic efficacies of HepG2, PLC/PRF/5 and MHCC97H cell lines including both the parental cells and spheroid cells in nude mice, and drew an inference that the spheroid cells exhibit high tumorigenicity in vivo (Also shown on Page 10-11, Page 14 and Fig. S1 in the revised version).

3. The observations on resistance to chemotherapeutic agents should be extended to include other common drugs with different mechanisms of action, eg, doxorubicin (ABC transporter blocker). Is there preferential survival of candidate CSC markers in this assay (easily tested by IHC for CD133, CD13, CD44 etc)?

Response:

We agree with the comment and we have tested the sensitivity of spheroid cells to other 4 drugs in addition to cisplatin. The PLC/PRF/5 spheroid cells exhibited general resistance to 5-Fu, gemcitabine, mitomycin and sorafenib in the treatment for 36 h. Compared with the parental PLC/PRF/5 cells, the survival rates of PLC/PRF/5 spheroid cells were higher under 200 μmol/L, 400 μmol/L of 5-Fu (1.60-fold, 1.98-fold respectively); 5 mmol/L, 10 mmol/L of gemcitabine (1.99-fold, 2.49-fold respectively); 0.5 μmol/L, 1.0 μmol/L of mitomycin (1.24-fold, 2.33-fold respectively); and 6 μmol/L, 12 μmol/L of sorafenib (2.07-fold, 15.21-fold respectively) (Shown on Page 9-10, Page 14-15 and Fig. 2 in revised version).

CD13, as a new reported liver CSC maker, has been referred in the Background
and Discussion parts in the revised version. Although we ordered CD13 antibody from oversea company for a long time, but it does not come up-to-date. So we compared the expression of candidate CSC markers CD133 and CD44 between the parental and sphered cells of HepG2 and MHCC97H by confocal immunofluorescent labeling, which is superior to IHC for observing the location of cellular antigens. Fortunately, CD44 expression was obviously enriched in HepG2 and MHCC97H sphered cells compared with their parental cells. CD44 is a polymorphic family of immunologically related cell surface proteoglycans and glycoproteins, normally takes part in cell-cell and cell-matrix adhesion interactions, which is involved in cancer cell migration, proliferation and metastasis. So the CD44 expression enrichment in sphered cells may account for their increased survival ability and tumorigenicity (Shown in Fig. S2 and Page 11 in Results, and Page 15 in Discussion).

4. The correlation between increased tumorigenicity and enhanced expression of candidate cancer stem cell markers in figure 4 is highly tentative. As a minimum, the Western blots need to be accurately quantified. However, it would be far more informative to demonstrate by IHC analysis that populations of cells co-expressing markers such as CD133 and CD44 etc are enriched in the spheres. CD13 should also be included here (See Haraguchi et al., JCI 2010,120:3326).

Response:

Thanks. According to your comment, we have quantified the bands of Western blots by software analysis, and showed in figure 4B.

As described above, we compared the expression of candidate CSC markers CD133 and CD44 between the parental and sphered cells of HepG2 and MHCC97H by confocal immunofluorescent labeling, and found that CD44 expression was obviously enriched in HepG2 and MHCC97H sphered cells compared with their parental cells, which may account for their increased survival ability and tumorigenicity (Shown in Fig. S2 and Page 11 in Results, and Page 15 in Discussion).

5. The link with aberrant activation of Notch signaling, although preliminary are
potentially interesting given the current interest in defining signaling pathways linked to maintenance of CSC function in-vivo. This could easily by functionally evaluated by blocking the Notch pathway in the sphere colonies with commercially-available gamma-secretase inhibitors.

Response:

Thank you for your good suggestion. We have added the contents in the Results Section on Page 12 and Fig. 5 in revised version. Blocking Notch pathway was performed by a gamma-secretase inhibitor MRK003. The secondary dissociated PLC/PRF/5 spheroid cells were treated with 10 µM MRK003 or DMSO control for 7 days. The decrease of activated Notch1 and downstream target genes DTX1 and Ep300 were confirmed by Western blotting. The sphere-forming of MRK-treated groups were significantly inhibited in comparison to DMSO-treated controls. The results indicated that the CSL-independent Notch signaling pathway might play an important role in liver CSCs and MRK003 could partly eliminate the stem-like cells.

Again, we are really grateful to editors and reviewers for pointing out many important issues. Now we believe that we have thoroughly edited the whole manuscript and that our responses are satisfactory.

Respectfully!

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