Author's response to reviews

Title: Doxorubicin-enriched, ALDHbr mouse breast cancer stem cells are treatable to oncolytic herpes simplex virus type 1

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

Thank you very much for giving us an opportunity to revise our manuscript entitled “Doxorubicin-enriched, ALDH<sup>hi</sup> mouse breast cancer stem cells are sensitive to oncolytic herpes simplex virus type 1” (Manuscript ID: 1165937510745165). We deeply appreciate the reviewer’s constructive comments on our manuscript and believe that the revisions have improved the quality of the manuscript substantially. We have submitted the revised manuscripts in two formats, with or without trace changes. Detailed responses to each of the points raised are given below.

Sincerely,

Xiufen Zhuang

Response to Reviewer #1:

1. The study was carried out based on their previous HSV1 vector published by Liu BinLei et al in the Chin J Oncol. I tried to find out the constructs of HSV1-GFP and HSV1-hGM-CSF unfortunately, ref. 33 cannot be found via PubMed, even though the title of the ref.33 does not show from internet. Surprisingly, HSV1 vector was used in the study whereas oHSV2-hGM-CSF was indicated in attached ref.33. I become more confuse about which type of HSV vector was investigated. Is it HSV1 or HSV2? To this point, I would suggest that authors should describe shortly about the constructs and characteristics of the vector, such as replication competent or defective, insertion sites and expression cassette, products of the insertion.

Response:
Ref. 33 was a paper from our lab published in Chin J Oncol., February 2012. In the previous study, the construction of both HSV1-hGM-CSF and HSV2-hGM-CSF was described, and their oncolytic potency was compared in vitro and in vivo.

We used HSV1 based viruses for the current study. The description for oncolytic HSV1 virus construction has now been supplemented in the Methods. Briefly, HSV1-GFP and HSV1-hGMCSF are replication competent. They were developed from HSV-1 strain 17+, in which the genes encoding infection cell protein 34.5 (ICP34.5) and ICP47 were removed, the expression cassette for human granulocyte–macrophage colony-stimulating factor (hGM-CSF) or green fluorescent protein (GFP) was inserted into the sites for ICP34.5.
2. What means br in ALDH^{br}? Is it shortcut from breast cancer and resistant to or other means?

Response:  
Br in ALDH^{br} is the abbreviation for bright, ALDH^{br} cells indicate cells with high ALDH1 activity. Please also see p4 line 16 of the revised manuscript.

3. p3 line 9 Immune-competent syngeneic mice p7 line 10 Female Balb/c mice  
Question: what kind of mice did you use in animal experiments? Is it immune-competent or immune-defective mice?

Response:  
All mice in our experiments were immune-competent female Balb/c mice. We have unified them in the revised manuscript.

4. p6 line 19 DMEM/F12 serum free medium (SFM) p9 line 7 SFM p9 line 9 FGM  
Question: To use DMEM/F12 SFM would be better than only use SFM. And again FGM can be changed to DMEM/F12 FGM.

Response:  
We have revised them in the manuscript according to your advice.

5. p10 line 14 a dose of 1X10^7 plaques  
Question: One plaque contains how many virus particles? Each batch has the same amount of virus particles per infection unit or different unit.

Response:  
According to Fields - Virology (Two Volumes) 4th Edition (2001, p41-45), “Two major types of quantitative assays for viruses exist, physical and biologic. Physical assays, such as hemagglutination, electron microscopic particle counts, optical density measurements, and immunologic methods, quantify only the presence of virus particles, whether or not the particles are infectious. Biologic assays, such as the plaque assay or various endpoint methods that have in common the assay of infectivity in cultured cells or in vivo, measure only the presence of infectivity, and they may not count all particles present in a preparation, even many that are in fact infectious.” “The plaque assay is based simply on the ability of a single infectious virus particle to give rise to a macroscopic area of cytopathology on an otherwise normal monolayer of cultured cells.” “A critical benefit of the plaque assay is that it measures infectivity, but it is important to understand that infectivity does not necessarily correspond exactly to the number of virus particles in a preparation. In fact, for most animal viruses, only a fraction of the particles, as few as 1 in 10 to 1 in 10,000, may be infectious as judged by comparison of a direct particle count with a plaque assay.”

The same batch of virus was used in all our experiments.
6. p10 line 12-16 mouse treated by HSV at day 5, 7, 9, 11, and 13...
Question: How did you treat control mice? What material or water did you inject to control mice? How did you demonstrate that the tumor killing effect is not due to mechanically destroy tumor rather than the function of the oncolytic HSV vector?

Response:
We treated the control mice with the solvent of doxorubicin and oncolytic HSV1 (NS intravenously and DMEM/F12 SFM intratumorally, respectively) (p11 line 3-5 of the revised manuscript).

Our experiments demonstrated that the tumor killing effect of OV was not due to the mechanical destroy, because the control group developed much larger-volume tumors even after mechanical injection with DMEM/F12 SFM.

7. p12 results line 9-12. Fig.1B ALDH\textsuperscript{br} cells have high mammosphere-forming ability
Questions: How do you demonstrate that the size of a mammosphere is related to tumor? (Because Normal stem cells can form mammosphere when the cells obtain a high level of colony stimuale factor)

Response:
We can not fully understand this question. Did you mean how do we relate mammoshere size to the tumorigenicity?

We have demonstrated that sorted 4T1 ALDH\textsuperscript{br} cells, which have larger mammosphere-forming ability, also have higher tumorigenicity in p13 and Table 1 of the primary manuscript.

8. p13 last line; Fig 2 Western blot
Question: not detected should be changed to not detectable.

Response:
We have revised it in the manuscript according to your advice.

9. p18 under discussion: line 3: high ALDH1 Question: is ALDH1 the same meaning as ALDH\textsuperscript{br}?  

Response:
ALDH\textsuperscript{br} cells refer to cells with high ALDH1 activity. According to the manufacture’s specification, “ALDEFLUOR is used to identify, evaluate, and isolate stem and progenitor cells that express high levels of aldehyde dehydrogenase (ALDH\textsuperscript{bright} or ALDH\textsuperscript{br})”.

According to Jan S. Moreb, et al, “it is widely presumed that this Aldefluor assay mostly measures ALDH1A1 isoyme activity due to the report that diethylaminobenzaldehyde
(DEAB) used in this assay is a specific inhibitor for ALDH1A1” (Chem Biol Interact. 2012 Jan 5;195(1):52-60.)

10. Fig 6A and Fig 6B
Question: Fig 6 A shows limited variation whereas 6B don’t show variation between various treatments.

Response:
4T1 model is considered to be very aggressive. So far, it’s very rare to see 4T1 tumor-free case after various treatments. The tumor size measurement and survival plot are two common means widely used for in vivo efficacy assessment for various tumor therapies.

In our study, the tumor size in Fig 6A was measured in 4 weeks after 4T1 inoculation, whereas the survival in Fig 6B was observed after 4 weeks, therefore they may not be comparable. Even though Fig 6B does not show significant variation between OV-alone and DOX+OV treatment groups, DOX+OV group had a longer median survival than OV-alone group (47 days for DOX+OV versus 40 days for OV-alone). Moreover, there is still statistically significance between the relevant groups in median survival (DOX versus Control, OV versus Control, DOX versus DOX+OV, p18 line 15-19 of the revised manuscript).

Reviewer #2:

This manuscript describes the isolation of cancer stem cells (sorting for ALDHbr cells) from the mouse 4T1 cell line. A number of papers have already described isolating 4T1 cancer stem cells. These cells are then used to test infectivity with oncolytic HSV and sensitivity to doxorubicin. Unfortunately, the actual sensitivity of the ALDHbr and lo cells to killing by doxorubicin or oncolytic HSV is never tested. The change in the % of ALDHbr cells after doxorubicin, and not oncolytic HSV treatment in vitro is not recapitulated in vivo for unknown reasons. While the manuscript focuses on cancer stem cells, the tumor model is generated from unselected, classically cultured 4T1 cells and it is not clear how or whether cancer stem cells in the tumor play any role in the efficacy seen with the combination treatment of doxorubicin and oncolytic HSV.

Response:
The reviewer's comment has given very good points regarding our manuscript. The cytotoxicity assay (MTT or CCK8) and CPE observation are the standard methods for the assessment of cell killing. However, we considered that the cytotoxicity assay would not be appropriate in our study as it is known that CSCs have the ability to self-renew and differentiate into multiple cell types. We understand that once the sorted ALDHbr cells are plated for cytotoxicity assay, they will inevitably differentiate into a mixture of ALDHbr and ALDHlo cells. Therefore, in our study we mainly checked the percentage of ALDHbr cells as an indirect but compromise indication for cell killing after different treatments. In addition, we evaluated the OV killing by observing CPE, in which the br and lo cells were infected
with OVs immediately after the sorted cells attached to the plate.

Due to the same reason that the sorted ALDH\textsuperscript{br} cells would proliferate and differentiate into a mixture of br and lo cells in vivo, we therefore used unselected, classically cultured 4T1 cells as the tumor model and measured the in vivo killing indirectly by checking the percentage of ALDH\textsuperscript{br} cells after different treatments.

**Major compulsory revisions:**

1. **In Fig 1C, is this the total number of mammospheres in a plate with 10\textsuperscript{5} cells? If so the efficiency is exceeding low. If not, what is the mammosphere efficiency (spheres/cells plated)? It is described as ± SEM, yet there are no error bars in the figure. How can SEM be determined from duplicate wells?**

   **Response:**
   The total number of mammospheres in a well was 5×10\textsuperscript{4} cells, as described in Methods, p7, line 7 of the revised manuscript. We had made a mistake in the number of cells for the mammosphere formation assay in the Fig 1C legend. We have now revised it (p30, line 11 of the manuscript).

   As for the low mammosphere-forming efficiency, this is because we only quantified the spheres larger than 25 μm in size. We did not present the number of spheres smaller than 25 μm because there was no significant difference in the number of spheres smaller than 25 μm between ALDH\textsuperscript{br} and ALDH\textsuperscript{lo} groups.

   As for the mammosphere efficiency (spheres/cells plated), it was 9 spheres/5×10\textsuperscript{4} cells for ALDH\textsuperscript{br} cells and 4 spheres/5×10\textsuperscript{4} cells for ALDH\textsuperscript{lo} cells when the mammospheres were 25–50 μm in size, 4 spheres/5×10\textsuperscript{4} cells for ALDH\textsuperscript{br} cells and 0 spheres/5×10\textsuperscript{4} cells for ALDH\textsuperscript{lo} cells when the mammospheres were larger than 50 μm in size.

   We misused SEM here, and have deleted it from the corresponding text in the manuscript.

2. **In Fig 3C, the ALDH\textsuperscript{lo} cells seem to be less well infected than the ALDH\textsuperscript{br} cells (ie., MOI=0.1), as opposed to similar, is this reproducible or due to image selection? What is the actual infectivity of ALDH\textsuperscript{br} and lo cells (%GFP-positive cells)? Previous studies with oncolytic HSV and 4T1 suggest that it is poorly permissive to HSV replication.**

   **Response:**
   Several images were taken at an indicated MOI and time, one typical image was selected. We repeated the experiment twice and the results were similar. As observed in Fig 3C (MOI 0.1 for 34 hours), the total number of ALDH\textsuperscript{br} cells was slightly more than that of ALDH\textsuperscript{lo} cells, and the GFP-positive cells appeared to be also slightly more than that of ALDH\textsuperscript{lo} cells. But the percentage of infected cells did not show significant difference in both subpopulations (38.8% versus 29.4%, respectively, p=0.0664, n=4–6). We have revised it in the manuscript (p16, line 1-2 of the revised manuscript).
In our experiments, 4T1 cells appear to be semi-permissive to HSV1 replication (Fig 3A), which is consistent with the result published by Mikihito Nakamori, et al. (Molecular Therapy, 2004).

3. The in vitro experiments (pg 15) don't measure cytotoxicity, so it is not possible to conclude whether oncolytic HSV kills any cells (unlikely at this early time point). What is the cytotoxicity of oncolytic HSV in the 4T1 cells (ALDH<sup>br</sup> or lo)?
How was the dose of doxorubicin selected and what is the cytotoxicity (survival/proliferation assay) of this dose in ALDH<sup>br</sup> and lo cells?

Response:
We partially agree with the reviewer’s comment that it’s hard to conclude OV killing without cytotoxicity assay. But OV infection of ALDH<sup>br</sup> or lo cells causing CPE in our study could also be considered as an evidence for OV associated tumor cell killing. We have replaced the word “sensitive” in the manuscript with “treatable” or “infectable”.

Because the sorted 4T1 ALDH<sup>br</sup> cells were able to differentiate rapidly after inoculation even with low FCS concentration, it was difficult for us to directly measure the cytotoxicity of OV or DOX to these sorted cells. Instead, we presented the percentage of ALDH<sup>br</sup> cells after OV and doxorubicin treatments.

The dose of doxorubicin was determined according to the in vitro DOX-induced CPE (p16, line 6-8 of the revised manuscript) and the in vivo pilot experiments (data not shown).

4. The explanation for the high %ALDH<sup>br</sup> in Fig 5B (pg 17) doesn't make sense as the tumors were harvested at day 11, closer to 1 week than to 3 weeks, and still much higher than in Fig 5C. What was the size of the tumors harvested in Fig 5B? OV treatment reduced the %ALDH<sup>br</sup> compared to DOX, yet the tumor sizes look the same in Fig 6A. Was there a difference in the DOX or OV sensitivity of ALDH<sup>br</sup> cells isolated from tumors versus in culture? Oncolytic HSV has been reported to induce a robust anti-tumor immune response in the 4T1 model, could this account for the effect on ALDH<sup>br</sup> cells?

Response:
The time of harvesting tumors for ALDH<sup>br</sup> examination after different treatments was at day 11, which was actually closer to 2 weeks. Fig 5C was to demonstrate the influence of primary tumor volume to ALDH<sup>br</sup> cells. We have attached the full experiment data for Fig 5C below. We did not exam the primary tumor volume at day 11 when ALDH<sup>br</sup> cells were detected, but we exam it at day 12. As shown in Fig 6A, the mean primary tumor volume at day 12 was as follows: 145.056 mm<sup>3</sup> for DOX+OV, 182.948 mm<sup>3</sup> for DOX alone, 275.363 mm<sup>3</sup> for OV alone, and 501.649 mm<sup>3</sup> for control, respectively.
As the reviewer pointed out, there was an unexpected change in the percentage of ALDH$^{br}$ cells between Fig 5B (32.10% ALDH$^{br}$ cells for 2 week tumor) and Fig 5C (20.22% ALDH$^{br}$ cells for 3 week tumor). We speculate this inconsistency may be due to that Fig 5B and Fig 5C were from two experiments with variable conditions (animal batches, experiment deviation, etc.). We will further explore this in future study.

We did not compare 4T1 ALDH$^{br}$ cells isolated from tumors versus in culture for their sensitivity to DOX or OV.

Although it has been reported Synco-2D and FusOn-H2, the fusogenic oncolytic HSVs induced potent antitumor immunity” (Mikihito Nakamori, et al. 2004, Molecular Therapy and Hongtao Li, et al. 2007, J Gene Med), the oncolytic HSV1 used in our study, seems not to be able to induce significant ALDH$^{br}$-specific immune responses (our preliminary data not shown). We will explore this matter further in future study.

5. There are a number of papers describing oncolytic HSV treatment of 4T1 tumors, especially from the lab of X. Zhang. The efficacy of HSV1-hGM-CSF should be discussed in comparison to these other papers, including ref 25.

Response:
We have revised the relevant paragraph in the discussion as the reviewer suggested.

According to the above two papers (Mikihito Nakamori, et al. 2004, Molecular Therapy and Hongtao Li, et al. 2007, J Gene Med), the two fusogenic oncolytic HSVs (Synco-2D and FusOn-H2) have substantial oncolytic effect to 4T1 cells and induced strong anti-tumor immune response against primary and metastatic mammary tumors in vivo, whereas the nonfusogenic oncolytic HSV1 (Baco-1) had limited effects on the same primary and metastatic tumors. In addition, a fusogenic HSV1 (OncdSyn) had similar anti-tumor activity (ref. 25).

Similar to these papers, our oncolytic HSV1 was able to reduce the primary tumor volume effectively with slight side effect. Also consistent with our study, 4T1 model is difficult to treat and no tumor-free case has been observed after oncolytic HSV treatment in these
papers. In contrast to the above papers, no significant anti-ALDH\textsuperscript{br} cell immune response was observed in our preliminary experiment. This may be due to that our oncolytic HSV1 is not fusogenic. Nevertheless, the main difference between our study and others is that we focused mainly on the oncolytic HSV1 targeting to CSCs (ALDH\textsuperscript{br} cells) and the combination of OV with chemo-agent doxorubicin for tumor therapy.

**Minor Essential Revisions:**

1. A description of the oncolytic HSVs should be provided, including their mutations and transgene structure. The reference listed (33) is not in PubMed, in English, nor full text available and the title indicates HSV-2 not HSV-1.

   **Response:**
   Ref. 33 was a paper from our lab published in Chin J Oncol., February 2012. In this paper, the construction of both HSV1-hGM-CSF and HSV2-hGM-CSF was described, their oncolytic potency was compared in vitro and in vivo. Briefly, HSV1-GFP and HSV1-hGMCSF are replication competent. They were developed from HSV-1 strain 17+, in which the genes encoding infection cell protein 34.5 (ICP34.5) and ICP47 were removed, the expression cassette for human granulocyte–macrophage colony-stimulating factor (hGM-CSF) or green fluorescent protein (GFP) was inserted into the sites for ICP34.5. The description has been added in the Methods of the revised manuscript.

2. This is not really a metastatic breast cancer model (pg 10 title), but a subcutaneous implant model.

   **Response:**
   Thank you for your suggestion. We accept your comment and have revised the manuscript accordingly.

3. There have been a couple of recent papers that describe 4T1 cancer stem cells (Matilainen, H et al, '12), including with aldefluor (Park SJ et al, '11), which should be referenced.

   **Response:**
   We have referenced the two papers in p5 line 1 of the revised manuscript.

4. Describe in Fig 1A legend what the 2 left panels represent. What percent of cells are ALDH\textsuperscript{br}?

   **Response:**
   As shown in the 2 left panels of Fig 1A, the first gate (P1) chooses the cells with good status from the total cells and excludes cell debris according to their FSC & SSC values. We have revised Fig 1A legend accordingly.

   In Fig 1A for sorting by flow cytometry, the percent of ALDH\textsuperscript{br} cells was 3~5%. We have...
added the percent of ALDH$$^{br}$$ cells in the Fig 1A legend.

5. "GFP was detected in cells of both the outer and inner spheres" (pg 14). There is no way to know whether the fluorescence is coming from inner or outer cells without confocal microscopy or sectioning. Therefore this conclusion is not supported by the data. At an MOI=1, the number of infected cells looks very small, so that the mammospheres are not "effectively" infected.

Response:
We have revised the sentences containing “outer” or “inner” from the manuscript, as shown in p15 of the revised manuscript.
We agree with the reviewer’s comment and have deleted "effectively" from the manuscript.

6. Fig 5A legend does not indicate what the upper panels (red) are and the labeling in the figure is small and not very clear. The percent of CD45- cells in each of the groups should be indicated on the figure or in the figure legend.

Response:
In Fig 5A, the upper panels are the gates (R2) to exclude APC anti-mouse CD45+ leukocytes. We have revised the Fig 5A legend accordingly.
We have also magnified the labeling in Fig 5A (as shown below and in the revised manuscript) and indicated the percent of CD45- cells in the figure legend according to your advice.

7. Why was OV administered after DOX (Fig 6)?

Response:
As shown in p21 line 4-5 of the revised manuscript, we chose this schedule mainly because “The majority of the non-CSCs were first eradicated by chemotherapy and then the residual CSCs were killed by oncolytic HSV1.”

8. The title is misleading and should be changed, "doxorubicin-enriched, ALDH$$^{br}$$
mouse breast cancer cells" were never examined and the "sensitivity" only relates to infection and not killing.

Response:
We accept the reviewer’s comment and have replaced “sensitive” with “treatable”.

9. The supplemental material is not referenced in the text and therefore is not really supplemental but additional. Why is it not included in the manuscript?

Response;
The supplemental material was referenced in p20 line 10 and p21 the last line 3, respectively.

Discretionaly Revisions:
1. It is not clear that the differences reported between ALDH\textsuperscript{br} and lo cells in vitro are related to the results in vivo. Even if OV does kill ALDH\textsuperscript{br} cells, it is not sufficient to cure the mice. Is there a difference in tumors generated from ALDH\textsuperscript{br} cells (Table 1) versus unsorted 4T1 cells (as in Fig 5, 6)?

Response:
Because the sorted ALDH\textsuperscript{br} cells will differentiation quickly when injected s.c into mice, we did not compare the difference in tumors generated from ALDH\textsuperscript{br} cells versus unsorted 4T1 cells.