Author's response to reviews

Title: CD133 expression in chemo-resistant Ewing sarcoma cells

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Author's response to reviews:

Thank you very much for your letter concerning our manuscript (3824467663028310): “CD133 expression in chemo-resistant Ewing sarcoma cells” by Xiaohua Jiang, et al. We appreciate the time and effort of the reviewers and respectfully herein submit a revised version of the manuscript. We appreciate the opportunity to improve our manuscript and believe that we have thoroughly and adequately addressed the comments of the reviewers.

Please note that since original submission the corresponding author (ER Lawlor) has moved to the University of Michigan. Updated contact information has been included on the title page.

Detailed response to the reviewers comments are below. We thank you for your time and look forward to a favourable response in the near future.

Sincerely,
Elizabeth Lawlor and Xiaohua Jiang

Response to Reviewers

Reviewer 1:

Major essential revision:
1. First, the novelty that the manuscript provides to the Ewing’s sarcoma cancer stem cell field is limited, since a recent article, published in Cancer Research in 2009 (Ref 16), has already identified the existence of a cancer stem cell population in Ewing sarcoma using freshly isolated primary tumor specimens. The novelty of the present work is therefore restricted to the discovery of a Ewing sarcoma cell line containing a CD133 positive population of cells that seems to retain the cancer stem cell hierarchy where the CD133 positive cell fraction
displays increased chemo-resistance.

While it is true that CD133 has been identified as a cancer stem cell marker in Ewing’s sarcomas by Suva et al in 2008, it should be noted that, to our knowledge, no report has to date addressed the association of drug resistance and putative cancer stem cell marker(s) in Ewing’s sarcoma. The purpose of this study was to evaluate the potential utility of PROM1/CD133 as a marker of chemoresistance. Assessment of stemness and tumorigenicity were secondary aims of the study. Our results demonstrate that in some tumors and cell lines high frequencies of PROM1/CD133 expressing cells are associated with drug resistance. In particular, we identify a case of primary drug resistant disease and a cell line, STA-ET-8.2, that displayed a CD133-defined cellular hierarchy with CD133+ cells representing a relatively drug-resistant, tumorigenic fraction. In this regard, we strongly argue that our study is indeed novel in that it shows, for the first time, that high levels of PROM1/CD133 are associated with resistance to chemotherapy in at least some cases of Ewing’s sarcoma.

2. The second major problem is the use of inappropriate methods to test the expression of CD133 in order to conclude that it is an “inconsistent” marker of chemoresistant tumor-initiating cells. Indeed, only a specific glycosylated epitope of CD133 is related to stem cells and cancer stem cell biology in all published studies thus far, and this epitope is recognized only by the AC133 monoclonal antibody (Miltenyi). In their work, the authors use RT-PCR and Quantitative Real-Time PCR to assess the expression of CD133 in primary tumors and cell lines (Figure 1A, 1B and 2A), but it is obvious that the results obtained cannot give any significant information regarding the expression of the stem cell-related epitope of CD133, or the presence of cancer stem cells in the tested samples. Moreover, the Western Blot and Immunohistochemical analysis (Figure 1C, 2A and 2B) performed by the authors in order to test the expression of CD133 in only two primary tumors and the different cell lines, are performed using Santa-Cruz and Abcam polyclonal antibodies, instead of the antibody recognizing the glycosylated, stem cell-related, AC133 epitope (Miltenyi). This approach hampers any identification or quantification of CD133-positive cancer stem cells in all the samples tested, and the final conclusions of the work based on these experiments are therefore flawed. The only possibility to investigate a broad panel of primary tumors for the presence of CD133 positive cancer stem cells is immunohistochemical analysis of frozen sections and the concomittant isolation of tumor cells from fresh surgical specimens of Ewing sarcoma using the AC133 antibody, followed by assessment of the tumorigenic potential displayed by the two cellular fractions. Without these experiments, the authors should at least reformulate their conclusions about the presence and percentage of chemo-resistant cancer stem cells in primary Ewing sarcoma and clearly discuss these limitations in the abstract and the discussion sections.

We thank the reviewer for pointing out these important issues for clarification. Almost all CD133-related studies carried out to date have made use of the AC133 and AC141 mAbs to identify and purify CSC populations, however, there are several drawbacks by using these antibodies specific to glycosylated epitope
of CD133 in every biological analysis. First, although the AC133 and AC141 mAbs are widely reported to bind glycosylated epitopes on CD133, the exact location of the modified amino acid residues on CD133 targeted by AC133 and AC141 mAbs has not yet been described. Thus, it is possible that the AC133 and/or AC141 mAbs recognize glycosylated epitopes on molecules rather than CD133. Secondly, several alternatively spliced forms of CD133 have been described in both human and mouse. Although currently unknown, alternatively spliced CD133 isoforms lacking the AC133 or AC141 epitopes could exist. Additionally, several studies have demonstrated that the AC133 and AC141 epitopes can be downregulated independently from the CD133 protein or mRNA. Thus, given these complexities, it may be incorrect to call AC133 or AC141 epitope-negative cells “CD133-negative” without proper verification of CD133 protein or mRNA levels.

As a first step to examine the expression of CD133 in both primary ESFT and established cell lines, we used RT-PCR to determine the presence of human CD133 (human PROM1) transcript (Fig1A, 1B and Fig 2A). Due to the uncertainty surrounding the target epitopes and specificity of the AC133 and AC141 mAbs, we used western blot and immunohistochemistry with antibodies targeting peptide instead of epitope (Santa cruz, C-19; Abcam) to evaluate the expression of CD133 at a protein level (Fig 1C, Fig 2A and 2B). Based on the finding that ESFT do express CD133 at variable level, we used flow cytometry to more precisely define the proportion of CD133+ vs. CD133- cells by using antibody specific for the CD133 epitope that has been used to characterize and isolate stem cells.

Given the fact that the AC133 and AC141 epitopes have been extensively used as markers for purifying CSCs in various tumors, it is likely that the glycosylation status of CD133 may be a more specific marker of the CSC phenotype than CD133 protein levels. In this respect, we agree with the reviewer that the existence of CD133 transcript and protein may not be sufficient to identify the CD133-positive cancer stem cells in the primary tumor samples. Therefore, in this revised manuscript we reformulate our conclusions and further discuss the limitations of our studies. In particular, we stress the need for studies that assess transcript, protein and glycosylated protein levels simultaneously in a prospective cohort of patients.

3. For the characterization of the STA-ET-8.2 cell line, the authors use the AC133 antibody to assess the percentage of CD133 positive cells, and to further sort the two fractions by FACS analysis, prior to soft agar, tumorigenic and chemo-resistance assays. The results obtained using the STA-ET-8.2 cell line are therefore interesting since they offer the possibility to use a cell line to further investigate the biological properties of the Ewing sarcoma cancer stem cells. However, the tumorigenic assay should be improved: the authors use 5x106 tumor cells to test the difference in tumorigenic potential between the unsorted, CD133 positive and CD133 negative populations, and find a significant difference between the two sorted fractions but also a surprising decrease in tumorigenicity compared to the unsorted cells. The number of cells used for this experiment is very high and begs the question what is the minimal amount of cells needed to
obtain a tumor from the unsorted cells? It would be interesting to repeat the experiments using a more permissive animal model (such as NOD-SCID Common-g KO), sorting the cells using only magnetic microbeads (that the authors use to enrich CD133 cells prior to FACS analysis), and performing the subcutaneous or orthotopic injections using limiting dilutions of the cells, to determine the minimal amount of CD133 positive or unsorted cells needed to obtain tumor formation.

In the same result section, the comparison made by the authors between the percentage of CD133 positive cells in the tumor xenografts and in the negative fraction prior to injection into NOD-SCID mice is arbitrary, since the former assessment is performed using a polyclonal antibody and the latter using the monoclonal AC133 antibody that recognizes only the cellular subpopulation expressing the stem cell-related glycosylated epitope of the CD133 protein.

We completely agree with the reviewer that the serial transplantation experiments in animal models remains the golden standard assay in characterization of cancer stem cell and it will be very interesting to compare the ability of CD133+ vs. CD133- cells in generating xenograft tumors using well-defined methodology. Our findings using 5x106 cells/injection are reported merely to demonstrate that the STA-ET-8.2 cell line does indeed demonstrate differential tumorigenicity between CD133+ and CD133- cells in vivo and these data corroborate our in vitro soft agar studies. The precise tumorigenic potential of these respective fractions will need to be determined in future studies that are designed to evaluate tumor-initiating cell frequency and we now state this expressly in the revised manuscript. As mentioned above, the present study was designed primarily to investigate the potential association between CD133 expression and chemosensitivity in primary tumors and cell lines and the potential existence of a cellular hierarchy in STA-ET-8.2 cells.

It has been our observation that the Miltenyi CD133 monoclonal antibody is generally of lower quality than the Abcam polyclonal CD133 antibody in immunohistochemical staining. Moreover, by comparing the results of immunostaining experiments done with Abcam polyclonal antibody to the FACS results obtained with the glycosylation-specific AC141 mAb in ESFT cell lines (Fig 2B&C), we conclude that these two antibodies correlate well in detecting CD133 positive cells. Consistent with our own findings, the Abcam antibody has been extensively used for immunofluorescence staining to identify CD133 positive cells by other groups (Dubrovskα A et al. PNAS. 2009; Walton NM et al. et al., 2008).

4- Finally, in the determination of the chemo-resistance it would be important to test the sensitivity of the CD133 positive and negative cells to the different chemotherapeutic drugs alone and in different combinations, instead of testing only the three drugs together.

In response to the very reasonable suggestion of the Reviewer, we include additional experiments in this revised manuscript. Our results showed that CD133+ STA-ET-8.2 cells displayed more resistance to Doxorubicin, Etoposide
or Vincristine alone, as well as in combination. These data have been added to Figure 6.

Minor Essential Revisions

1- In the result section there is an inversion between Figure 1B and 1C in the text.

We apologize for the confusion caused by the mislabeling. The error has been corrected in the revised manuscript.

Reviewer 2:

Major Compulsory Revisions:

1. The authors used CD133/2 for FACS and CD133/1 for magnetic bead labelling and separation preceding FACS. Is it mandatory to use two different antibodies and does the preselection with CD133/1 allow for a direct comparison of the FACS-sorted populations generated with CD133/2?

Is the CD133/2 antibody from Miltenyi the same clone (293C3) that was employed by Suva et al. (Cancer Res. 2009;69:OF1-6)? The authors must resolve these issues

Since MACS-separated cells might be saturated with the antibody (CD133/1), it is recommended by the manufacturer to use an alternative antibody recognizing the second epitope of CD133 (CD133/2) when performing subsequent analyses of cell separation. Thus, we used CD133/2 in the FACS experiment when evaluating cell sorting efficiency. We have added this important detail to the methods section.

This protocol was identical to that used by Suva et al., who used the same CD133/1 (293 C3) antibody from Miltenyi to separate the cells by MACS and then analyzed the quality of separation by CD133/2.

2. Two transcripts of different length have been described to encode CD133. Do the PCR primers which the authors used detect both mRNA transcripts? Why were some samples completely negative in the RT-PCR shown in Fig. 2A (even a few cells would be expected to give a positive signal in a standard RT-PCR assay). For comparison: Suva et al reported 8/8 patients to be positive by (presumably less sensitive) protein-based detection methods like flow cytometry/immunocytochemistry! Is this due to a sensitivity problem of the PCR method applied in this study?

The Taqman QPCR assay was purchased from Applied Biosystem (Hs01009261-m1), and detects the CD133 (PROM1) cDNA sequence from 1115 to 1221. This assay detects both AC133/1 and the AC133/2 isoform, which lacks exon 4 resulting in a deletion of nine amino acids in the N-terminal extracellular domain as reported by Yu Y et al (JBC., 2002). This has been added to the methods section. With respect to the RT-PCR data in cell lines, the data presented in Fig2A are a representative gel image showing 30 cycles of amplification. The transcripts can be detected by gel electrophoresis when the
cycle number is increased to 35 cycles. This important detail has been added to the figure legend.

With respect to the frequency of CD133+ cells in Ewing’s cell lines, our FACS analyses identified CD133+ cells in all ESFT cell lines tested, although the frequency of these cells varied substantially, ranging from only 2% to nearly 100%. Similarly, we do not think that our primary tumor data are inconsistent with those of Suva et al. Quantitative Taqman PCR of primary tumor samples demonstrated a wide range of expression with Ct detection limits ranging from 20 cycles to undetectable after 40 cycles (5 cases). Normalization of CD133 expression to GAPDH (or ACTIN) in each sample results in expression values ranging from 0 to 12.5%. Median expression in these 48 cases was 0.06% relative to GAPDH. These data are not inconsistent with Suva et al. who, by immunohistochemical staining of 5 cases, found CD133 positive cells to be exceedingly rare in primary tumor sections. In 3 cases (for which they had abundant tissue) they were able to quantify the frequency to be from 4-8% of cells using FACS. The RNA samples for our gene expression studies was extracted from frozen tissue sections obtained from minimal closed needle biopsy specimens. Therefore, it is not surprising that in some cases we would be unable to amplify the CD133 transcript. We have added this possibility of “false negatives” to the discussion.

3. The PCR data were normalized with only one housekeeping gene (GAPDH). Ideally, data for PROM1 should be normalized with at least two HKGs to minimize possible variations among samples.

We used both GAPDH and ACTIN to normalize the data in our Taqman analysis. Since there was no difference when the data were normalized to either gene only GAPDH is shown. This point has been added to the methods and results.

II. Minor Essential Revisions:

1. The labelling of the ordinata and abscissa in Fig. 1 panel A needs to be changed (ordinate: Expression, abscissa: Number of tumors). According to the first paragraph on page 10 (Results) and the corresponding legend this panel is supposed to show the expression of 48 tumor specimens, however I see only 14. What does the numbering on the abscissa refer to? The 11 cases in which PROM1 was readily detected? This needs to be rectified.

We apologize for the confusion. The figure is a frequency histogram in which tumors are grouped together according to range of gene expression (e.g. 0-1%, 1-2%, 2-3%, ..., 12-13%), resulting in 14 groups of gene expression with 1 or more samples in each range. The # of tumors in each of these quantitative categories is on the ordinate/y-axis and the corresponding expression level range is on the abscissa/x-axis. As shown, the vast majority (N=37) of tumors had PROM1 levels of expression ranging from 0-1% (relative to GAPDH). A total of 11 tumors had expression levels >1% and 4 of these were >3%. To clarify this data we have altered the figure legend to be more descriptive and have changed the labeling of the ordinate to include the range.
2. Page 5, first paragraph, last sentence: the last part of this sentence should be changed to: ...established cell lines and we conclude that CD133 is inconsistent as a marker of ...

The sentence has been changed according to the suggestion of the reviewer.

3. Figure 6: Do results were similar when the cells were exposed to the cytotoxic drug combination for shorter periods of time?
To strengthen their conclusions on the role of CD133 expression for chemoresistance in the STA-ET-8.2 cells, corresponding results from one of the other ESFT cell lines should be depicted as part of panel A.

We determined the chemo-resistance of CD133+ Vs. CD133- cells for 24-96 hours. The difference between CD133+ and CD133- populations starts to show at 48 hours, and is most prominent at 96 hours. To strengthen our conclusion that CD133 expression is not associated with chemoresistance in all ESFT cell lines, we have included the data of CD133+ vs. CD133- TC71 cells (Fig. 6E).

4. Page 11, first paragraph: Figure 1B is incorrect as these data (frequency of CD133+ cells in tumor tissue section) are shown in panel C. "Fig. 1C" should be moved either behind "frequency of CD133+ cells" or behind "induction chemotherapy" to precede the conclusion drawn here. The term "data not shown" should be inserted behind "the level of PROM1 (transcripts)" because mRNA data were not depicted in this figure.

We thank the reviewer for noting these errors in our original manuscript. We have corrected these errors in the revised manuscript.

5. Page 14, second paragraph: Did the authors, as a control, try to expand in culture clonally derived spheres also from single CD133- cells to see whether they can give rise (spontaneously) to CD133+ progeny?

We thank the reviewer for this suggestion. We have not performed this assay but agree that it would be informative to demonstrate that the hierarchy is truly "one-way". Certainly, studies of other tumor types would suggest that CD133- cells will be unable to generate CD133+ progeny. In keeping with this, CD133- STA-ET-8.2 cells did not give rise to tumors in vivo while CD133+ cells generated tumors comprising both cell types. As discussed in response to reviewer one’s comments above, we agree that there are many more experiments that need to be done to definitively address the issue of tumorigenic potential of cell fractions in the STA-ET-8.2 cell line. These important studies are outside the scope of the current report which is focused more on determining if there is an association between CD133 expression and drug resistance in Ewing's sarcoma.

6. Page 17: Since the process of FACS-sorting negatively affected the tumor-initiating capacity of STA-ET-8.2 cells, did the authors repeat this animal experiment with cells purified with magnetic beads?

These very worthwhile studies have not yet been performed. We agree that
many more experiments outside the scope of the current study are necessary to address the issue of how FACS procedure itself might affect the tumorigenicity of cancer cells.

III. Discretionary Revisions

1. Abstract (first sentence on page 3): This sentence is incomplete. It should be stated for what features the authors compared the CD133+ and CD133- negative cells (and have found no differences).

As suggested by the reviewer we have revised this sentence to be more complete.