Author's response to reviews

Title: YB-1 regulates Sox2 to coordinately sustain stemness and tumorigenic properties in a phenotypically distinct subset of breast cancer cells

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Version: 2 Date: 14 April 2014

Author's response to reviews: see over
Re: Revised original article submission – MS #1128518541179382

Dear Dr. Carnero:

We are happy to re-submit our revised manuscript entitled “YB-1 regulates Sox2 to coordinately sustain stemness and tumorigenic properties in a phenotypically distinct subset of breast cancer cells” for publication in BMC Cancer.

We have carefully considered all three of the reviewers' valued comments and suggestions. We have addressed all of their concerns point by point below and have revised the manuscript and figures as suggested. We thank the reviewers and journal for their time and consideration of our study.

We look forward to hearing from you.

Sincerely yours,

Raymond Lai
Dear Dr. García-Heredia,

We thank you for your valuable insights and suggestions. We have addressed all of your concerns as detailed below in BLUE.

Sincerely,
Raymond Lai

REFEREE #1

Reviewer's report

Title: YB-1 regulates Sox2 to coordinately sustain stemness and tumorigenic properties in a phenotypically distinct subset of breast cancer cells

Version: 1 Date: February 2014

Reviewer: José Manuel García-Heredia

Reviewer's report:

The manuscript explores the role of YB-1 as a regulator of the expression of Sox-2. In this article, the authors make an extensive characterization of the effects of YB-1 on the expression of Sox-2, and also, on the expression of other genes, like CCND1 or ITGA6, related to YB-1 or Sox-2. The manuscript shows that, in fact, YB-1 regulates the expression of Sox-2 in breast cancer cells. However there are some parts that need a review, before accepting it.

- Minor essential revisions:

1. In Methods section the authors describe two different siRNA species, namely as #1 and #2. However, with the exception of a supplementary figure (number 4), siRNA #2 is not used in the article, and they obtained better results only with siRNA #1 (“YB-1 siRNA denotes YB-1 siRNA#1 throughout the manuscript and figures”). However, in Figure 6 they used siRNA #2. The authors should consider the rewriting of “siRNA knockdown of YB-1” section. In addition, supplementary figure 4 should be modified, including also the results obtained with siRNA #1, or modifying the figure title.

REPLY: We have added the following statement to clarify the use of YB-1 siRNA #2 in our studies in the Methods section as suggested: “We have employed the use of 2 unique siRNA sequences targeted against YB-1. We have primarily used YB-1 siRNA#1 throughout the study as we have achieved successful and consistent knockdowns with this sequence in our laboratory and previous work done by the first author [25]; as well, it is the recommended validated sequence from the manufacturer. We have incorporated the use of YB-1 siRNA#2 in our study to validate the findings of YB-1 siRNA#1. In the mammosphere culture condition, we found that the YB-1 siRNA#2 sequence produced a more robust sustained knockdown 10 days post-transfection and thus we reported the results using the YB-1 siRNA#2 sequence. YB-1 siRNA denotes YB-1 siRNA #1 throughout the manuscript and figures.” We have modified the
2. In Results section, the first paragraph describes the regulation of Sox-2 by YB-1. The authors should describe the differences between “parental” (I supposed that they referred to BC cells not treated with siRNA) and “unsorted” (BC cells treated with siRNA) in a better way. Also, regarding to Figure 1, Panel B is not described until section 4, making necessary to include at least a commentary in section 1, when Figure 1 is described, or including it directly in Figure 4. Finally, regarding to Figure 1C, it is hard to see differences in Sox2 levels for ZR751 cells, although the authors wrote “YB-1 knockdown also induced an up-regulation of Sox2 protein expression…”. This result is only clear for MCF-7 cells.

REPLY: We have incorporated the following sentence for clarification in the Results section as suggested: “The “Unsorted” cells are the parental cells that have been stably infected with the Sox2 reporter but have not been purified or sorted into RU and RR cells.” We have clarified the reference to Figure 1B in section 1 of the Results as suggested. And yes, the up-regulation of Sox2 protein expression in ZR751 cells in Figure 1C is smaller compared to the MCF7, which is reflected in the sorted RU and RR populations also in Figure 1B.

3. When the authors refer to phospho-YB-1(Ser102), they wrote “is expressed higher...”. However, due to the role of phosphorylation as a post-translational modification, is not related with protein expression. Please, rewrite.

REPLY: We have corrected that to “elevated” levels of phospho-YB-1Ser102.

4. Figure 4B shows “relative luciferase activity”, but it lacks from a reference that represents 100%. In addition, assay with siScr and siYB-1 for RR MCF7 cells exhibit differences that does not seem significant.

REPLY: In Figure 4B, due to the spread of the luciferase values, 1 cannot be seen from that graph. We have added notes in the Figure legend to clarify the reference for all other values. Yes, the up-regulation of luciferase in MCF7 RR cells was not statistically significant, and we have added the following sentence in the Results to clarify: “The small increase seen in the MCF7 RR cells (Figure 4B) could be due to the innately high luciferase activity in that cell population.” Thus we have shown extensively in Figure 5 through other experiments that Sox2 transcription activity is up-regulated.

5. Figure 5B (left) and Figure 6C show the same lack of a reference for luciferase activity.

REPLY: In Figure 5B and 6C, due to the spread of the luciferase values, 1 cannot be seen from that graph. We have added notes in the Figure legend to clarify the reference for all other values.

6. At the beginning of discussion section, the authors refer again to “functional interaction” between Sox-2 and YB-1. Although it is clear that YB-1 is involved in Sox-2 expression, there are no data that allow to authors assume a direct interaction between both proteins, so the last sentence in the first paragraph of
the discussion should be rewrite. Also, probably YB-1 is involved in Sox-2 regulation not only in BC cells but also in all cells where both proteins are expressed. The assumption of a regulation only in BC cells is inaccurate.

REPLY: We have modified the last sentence in the first paragraph of the Discussion as suggested to: “We hypothesized that YB-1, another transcription factor important in stem cell biology and the pathogenesis of BC, regulates Sox2 in BC cells.” Further, we agree with the Reviewer that a regulatory relationship between YB-1 and Sox2 is not only in breast cancer cells. We have modified the Discussion to clarify this point: “It is likely that the regulatory relationship between these two important stem cell transcription factors is complex and the discrepancy between positive or negative regulation is cell type-specific.”

7. After an extensive reading of the article, I have not found the reason of working with two different cell lines (MCF7 and ZR751). The results obtained in all the experiments are very similar for both cell lines. I think that the authors should reconsider if it is necessary to show the experiments obtained with ZR571 cells, giving a good reason to use both cell lines.

REPLY: We have chosen to use both MCF7 and ZR751 cell lines throughout the manuscript to show that the relationship between YB-1 and Sox2 is not cell line dependent.

- Discretionary revisions:

1. In the background section the authors hypothesized about functional interactions in BC cells. However, I am not sure if they refer to interactions between YB-1 and Sox2 or other type of interaction.

REPLY: We have removed the term “functional interactions” in the manuscript as suggested.

2. Some abbreviations are missed: siScr (scrambled siRNA), ER-positive or ER-negative cells (Estrogen Receptor). Abbreviations should be defined the first time they are named.

REPLY: We have fixed this.

3. Figure 3 seems incomplete. In Figure 3A, the authors show how the levels of P-AKT(Ser473) decays with the LY294002 treatment. I miss the same result for RSK 1/2 and GSK3#.

REPLY: We chose to use the RSK and GSK3 inhibitors as RSK and GSK3 are directly upstream of YB-1 and phosphorylate YB-1 directly. Thus, we did not show the phospho-Akt and showed the phospho-YB-1 levels in Figure 3B.

4. In section “YB-1 knockdown induces differential gene…”, the authors refers Figure 5A twice in the same sentence. It sounds redundant.

REPLY: We have removed the redundancy.

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests: I declare that I have no competing interests.
Dear Dr. Martinez,

We thank you for your review of our study and your compliments.

Sincerely,
Raymond Lai

REFEREE #2

Reviewer’s report

Title: YB-1 regulates Sox2 to coordinately sustain stemness and tumorigenic properties in a phenotypically distinct subset of breast cancer cells

Version: 1 Date: 11 March 2014

Reviewer: Juan Martinez

Reviewer’s report:

Comments to:
YB-1 regulates Sox2 to coordinately sustain stemness and tumorigenic properties in a phenotypically distinct subset of breast cancer cells
Karen Jung, Fang Wu, Peng Wang, Xiaoxia Ye, Bassam S Abdulkarim and Raymond Lai
Submitted to BMC Cancer

The paper by Jung and collaborators shows that YB-1, a transcription factor previously found in embryonic stem cells and in mammary progenitor and tumor cells, and known to promote tumorigenesis, binds to the promoter region of Sox2, another transcription factor which is important for the pluripotency maintenance of embryonic stem cell. YB-1 silencing translates in a concomitant increase of Sox2 protein and/or mRNA in two mammary cell lines. Furthermore, since YB-1 shall be activated through phosphorylation at serine 102 by the kinases AKT, GSK-3b or RSK in order to get into the nucleus, treatment with chemical inhibitors of such kinases also increased Sox2 mRNA or protein in MCF7 cells, while AKT activation with IGF-1 translated in a Sox2 mRNA reduction. As previously shown by Lai’s group, depending on the responsiveness to a Sox2 reporter carrying two genes encoding for GFP and luciferase, cells can be divided into RR (responsive) or RU (unresponsive). Now they show that YB-1 regulates Sox2 reporter activity only in RR cells, and Yb-1 silencing induced a concomitant increase in Sox2 with enhanced tumorigenic properties in responsive cells but not in the unresponsive subpopulation.

The question posed by the authors is well defined, experimentation is appropriate and methodology well explained. Data in the paper are sound and figures have good quality. Discussion and conclusions are also well balanced and supported by the results. Limitations of the work, the use of chemical inhibitors, for instance, are clearly stated. Authors clearly acknowledge previous work in the field. Title and abstract accurately convey what has been found in the research. Finally, the writing is acceptable.
• **Major Compulsory Revisions**
  I do not propose any major compulsory revision.

• **Minor Essential Revisions**
  I do not propose any minor essential revision.

• **Discretionary Revisions**
  I do not propose any discretionary revision.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:** I declare that I have no competing interests
Dear Dr. Lleonart,

We are grateful for your time and detailed analyses of our study. We value your feedback and have addressed all of your concerns as detailed below in BLUE.

Sincerely,
Raymond Lai

REFEREE #3

Reviewer’s report

Title: YB-1 regulates Sox2 to coordinately sustain stemness and tumorigenic properties in a phenotypically distinct subset of breast cancer cells

Version: 1 Date: 12 March 2014
Reviewer: Matilde E. LLeonart

Reviewer’s report:

Strengths:
This is an interesting paper that reports a novel link between Sox2 and YB-1 transcription factors.

Weaknesses:
The manuscript needs more testing of the results presented here. Most of the results are represented in form of graphs (mRNA data, reporter experiments...). In this sense, several Western-blots are missing and pictures of mamospheres and soft-agar are needed to verify the data.

Major points:
1) Describe more clearly the differences between the two cell populations RU and RR instead of refer to a previous paper from the authors (first paragraph of results section).

REPLY: As suggested, the key differences between the RU and RR populations are now addressed in the Methods section and the Results section.

2) General Western-blot: Why the authors use the vinculin protein as the reference housekeeping protein instead of using β-actin?

REPLY: Vinculin is a cytoskeletal protein with a size of 120 kDa commonly used for loading (Liu et al. 2011, Nature Medicine). As Sox2 is 35 kDa and YB-1 is 50 kDa, β-actin at 42 kDa we chose to not overcrowd the western blot with bands in the same area for clearer analyses.

3) Fig. 1A and 1C: Why ZR751 cell line has so different basal levels of Sox2 in the parental cells in comparison to the unsorted cells? Aparently these are the same cell populations.
REPLY: Figures 1A and 1C were derived from separate experiments, separate western blots, and different exposures, which explains the small discrepancy between Sox2 levels. The ZR751 parental lines and the Unsorted lines have very similar high levels of Sox2 as we have previously reported (Wu et al. 2012, Cell Signal).

4) Figure 3: Fig. 3A and 3B: Western-Blot of Sox2 is missing. Moreover, while in most Western-blots Sox2 appears as a single band: Why a double-band appear in Fig. 3C-3D?

REPLY: We did not show the Sox2 bands in Figure 3A and 3B because we didn’t see any substantial increases in Sox2 levels with those inhibitors at those time points. LY294002 and SL0101 are not the most stable compounds in culture and thus we chose the time points of 6 hours and 24 hours based on the published half-lives. Thus, we focused on the CHIR compound with superior stability; and with a longer treatment, we saw an increase in Sox2 protein as shown in Figure 3C. We have early evidence in our lab that Sox2 is post-translationally modified just as it is in embryonic stem cells. We see the double band sometimes when the western blot gel is resolved more.

5) Figure 4: The authors claim at the beginning of the manuscript that the siYB-1#1 is the best siRNA for inhibiting the YB-1 protein. Why they sometimes show the siYB-1#2 or even only the siYB-1#2 in some figures (Fig. 4A both, Fig. 6 both, Suppl. fig. 6 only siYB-1#2, etc…).

REPLY: We have clarified the use of YB-1 siRNA #2 in our studies in the Methods section as suggested: “We have employed the use of 2 unique siRNA sequences targeted against YB-1. We have primarily used YB-1 siRNA#1 throughout the study as we have achieved successful and consistent knockdowns with this sequence in our laboratory and previous work done by the first author [25]; as well, it is the recommended validated sequence from the manufacturer. We have incorporated the use of YB-1 siRNA#2 in our study to validate the findings of YB-1 siRNA#1. In the mammosphere culture condition, we found that the YB-1 siRNA#2 sequence produced a more robust sustained knockdown 10 days post-transfection and thus we reported the results using the YB-1 siRNA#2 sequence. YB-1 siRNA denotes YB-1 siRNA #1 throughout the manuscript and figures.”

6) Figure 5: Fig. 5A results contrast with the results from Figure 4B. There is no details of how many experiments have been performed in figure legends or if this is the result of a single experiment done by triplicate, etc…Fig. 5B needs to show also results from ZR751 cell line as the whole figure does. Fig. 5D: ITGA6 western-blot is missing.

REPLY: In regards to the comment about the inconsistency between Figures 5A and 4B, in Figure 4B, we show that the MCF7 and ZR751 RR cells exhibit increased luciferase with YB-1 knockdown. Though it is not statistically significant in the MCF7 RR cells, we consistently see this pattern. We hypothesize that it is due to the innately high levels of MCF7 RR cells (~250x higher than RU) that we don’t see a further dramatic increase in luciferase, this is now detailed in the Results section. And accordingly, in Figure 5A, we see that the MCF7 RR cells do exhibit increased Sox2 transcription activity as shown by the increased downstream target NANOG transcripts.
In Figure 5B, we focused in on the MCF7 cells to demonstrate that Sox2 protein increases Sox2 activity because the luciferase data was not as clear as in the ZR751 cells. So we show that when you introduce Sox2 via an overexpression plasmid, you also see more NANO2 transcripts only in the MCF7 RR cells, which further confirms that Sox2 activity has been increased with Sox2 overexpression, despite the marginal increase in luciferase in Figure 4B. We have added these explanations in the Results section for added clarity as suggested.

In Figure 5D, the western blot for ITGA6 (alpha6-integrin) was not shown as the protein is generally low or barely detectable in ER+ cells and it is very difficult to detect in MCF7 cells. We will detail in statistical analyses the number of biological and technical replicates.

7) Figure 6: Pictures of mammospheres and soft-agar assays representative of the graphs are missing. Figure 6D needs to be accompanied by western-blot.

**REPLY:** Pictures of mammosphere and soft agar assays are not generally required for publication and thus we had omitted this in our original experiments. We have gone back and repeated the experiment to produce pictures for those assays for Figure 6, which has been incorporated into the new Supplementary Figure 6. Figure 6D cannot be accompanied by a western blot because we were collecting mammospheres for the experiment and the assay affords very small cell numbers, hence we chose to analyze the expression by qPCR.

8) Akt, RSK1/2 and GSK3β kinases are multi-faceted kinases and the inhibitors used undoubtly have a high cargo of non-specific effects (i.e. mutation of the Ser102 aa of YB-1 can prove that the role of the inhibitors is abolished and can provide clues about its role in Sox2 expression.

**REPLY:** Yes, due to some of the non-specificity of siRNA species and inhibitors, we have decided to use 2 unique siRNA sequence and 3 different inhibitors to show the same observed phenomenon between YB-1 and Sox2.

9) Results section “YB-1 regulates the Sox2 reporter activity only in the RR cell subset”: The sentence “When we performed the same experiment…(Figure 4B and 4c). This is only applicable to ZR751 cells but not MCF7 cells as in MCF7 cells results are not significant.

**REPLY:** Yes, the up-regulation of luciferase in MCF7 RR cells was not statistically significant, and we have added the following sentence in the Results to clarify: “The small increase seen in the MCF7 RR cells (Figure 4B) could be due to the innately high luciferase activity in that cell population.” And we have data shown in Figure 5 that indeed Sox2 transcription activity is up-regulated with Sox2 up-regulation either by YB-1 knockdown or Sox2 overexpression as downstream genes are up-regulated in the RR cells.

10) Result section “YB-1 knockdown induces differential gene expression patterns in RU and RR cells”: First sentence “As we have demonstrated…” is only applicable to ZR751 cells.

**REPLY:** Please see response to #9.

11) Result section “Up-regulation of Sox2 and its downstream targets…”. Show the results from Suppl. Fig 5 in Figure 6. The manuscript compares the results of
two cell lines in parallel.

**REPLY:** As Figure 6 summarizes data from only MCF7 cells, we put the ZR751 data in Supplementary for easier consumption.

12) How do you explain the controversy of your results with those from Fotovati et al., 2011? Do you only explain that by the cell type specificity? (i.e. did the authors try any glioblastoma cell line, etc...).

**REPLY:** Yes we have discussed the Fotovati 2011 paper in the discussion and we speculate it is due to cell type specificity. And no we have not yet looked at the YB-1/Sox2 interaction beyond breast cancer cell lines.

13) Discussion: The sentence “Specifically, following YB-1 knock-down stem cell genes NANOG and ITGA6...dramatically upregulated” is exaggerated.

**REPLY:** “Dramatically” has been removed.

14) Last paragraph of the discussion is speculative. It has to be supported by some experimental evidence or at least some references.

**REPLY:** The hypotheses were made in light of this study and our model directly. Further, our report of the interactions between YB-1 and Sox2 are novel and thus we were speculating future experiments that we could pursue. Because of this, there are no appropriate references or experimental evidence that we can draw upon.

**Minor points:**
1) Describe with more detail the manospheres culture.

**REPLY:** We had incorporated more details into the Methods section.

2) Describe more clearly in methods the differences among the cell lines used in the study.

**REPLY:** We have included clearer descriptions in the Methods section.

3) Results section.“YB-1 negatively regulates Sox2 expression in breast cancer”: The last sentence of first paragraph is not clear “In both MCF7 and ZR751 cells...”

**REPLY:** We have modified this to say: “In both the “Unsorted” MCF7 and ZR751 cells, YB-1 knockdown also induced an up-regulation of Sox2 protein expression (Figure 1C). The “Unsorted” cells are the parental cells that have been stably infected with the Sox2 reporter but have not been purified or sorted into RU and RR cells.”

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests: I declare that I have no competing interests