Author's response to reviews

Title: Musashi1 modulates cell proliferation genes in the medulloblastoma cell line Daoy

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Author's response to reviews: see over
Dear Ashleigh Manning,

Thank you very much for reviewing our manuscript entitled “Musashi1 modulates cell proliferation genes in the medulloblastoma cell line Daoy”. We also appreciate the constructive comments raised by the referees as they have definitely helped improving the manuscript. We would like to resubmit our paper with the revisions, additional data and clarifications requested.

We apologize for the formatting issues. We have revised the manuscript to conform to the journal style. We have incorporated a methods section in the revised version of the abstract and we have included the primers list as Table 1 rather than as additional file.

Below we address all the referees’ comments point-by-point.

Referee 1:

1.- The correction indicated by the referee in page 2 has been incorporated in the text.

2. - The correction indicated by the referee in page 3 has been incorporated in the text.

3.- We are confident about the knockdown specificity as we used the shRNAmir construct from the whole-genome retroviral shRNAmir library developed and validated by Hannon–Elledge (Silva et al., 2005) that is available for purchase from Open Biosystems. These shRNAs are designed based on real miRNAs, fact that contributes to prevent off target effects.


   b) http://www.openbiosystems.com/RNAi/shRNAmirLibraries/
4.- We have included the following explanation in page 8 “Since neurosphere cultures can be enriched in tumor re-initiating cells and Msi1 is a stem cell marker with a role in cell cycle progression (Battelli et al, 2006), the higher level of Msi1 detected in neurospheres indicated that Msi1 may contribute to Daoy cancer cell proliferation”. In response to referee’s comment, the monolayer cultures were about 80% confluent at the time of RNA preparation. We thus assume that cells in the monolayer cultures were not actively proliferating. We are incorporating this clarification in the background (page 4) and results (page 8) sections.


5.- As suggested by the referee, we are now using the terms “components” and “downstream targets” to establish a clear distinction, through the whole manuscript, between the genes encoding relevant effectors for the pathway signal transduction and those encoding targets of the pathway. We use the term “pathway related genes” when referring to both types of genes (“components” and “downstream targets”) simultaneously.

6.- We agree that Hedgehog signaling plays an important role in neurosphere formation in Daoy cells and it’s very reasonable to think that Msi1 depleted cells may be more susceptible to Hedgehog blockade than cells expressing higher levels of Msi1 because the pathway has been partially down-regulated. We appreciate this comment and it is now incorporated in the text. New data quantifying the differences in neurosphere size after cyclopamine treatment has been added to figure 5 (panel 5.b). We thank the referee for this suggestion, as our data is now more robust.

7.- As recommended by the referee, we have quantified the intensity of the bands in the western blots. We have used Adobe Photoshop CS3 Extended software. In the KD on average a 5-fold increase was detected in βIII Tubulin levels compared to control cells and a 8-fold decrease in Bcl-2 protein levels (p<0.05). This data has been incorporated in the revised version of the manuscript on page 12.

8.- This effect may be due to the inherent limitation of the neurosphere assay. As demonstrated by Singec et al., (2006) “neurospheres cannot be considered as clonal entities”; they are motile and tend to merge forming chimeric structures even under
“clonal” culture conditions. From our point of view, this may, at least in part, explain why the neurospheres are larger at higher cell densities. Though not shown, the effect was more evident at higher cell densities (i.e. 1000, 5000 cells/well). On the other hand, we cannot rule out the presence of certain secreted factors that may promote proliferation in a cell density manner.


Referee 2:

1.- We would like to indicate that from this experiment we do not conclude that “Msi1 may contribute to cancer progression” but to “cancer cell proliferation”. Following referee 1 suggestion, we’ve included in page 8 a sentence to better explain our thoughts; “Since neurosphere cultures can be enriched in tumor re-initiating cells and Msi1 is a stem cell marker with a role in cell cycle progression (Battelli et al, 2006), the higher level of Msi1 detected in neurospheres indicated that Msi1 may contribute to Daoy cancer cell proliferation”.

During the review process of our manuscript, Sureban et al., (2008) demonstrated Msi1 was involved in cancer cell proliferation, apoptosis inhibition and that was necessary for tumor growth of colon adenocarcinoma xenografts. Therefore our results are consistent with those obtained by Sureban et al. using a different model system.


2.- In response to the referee comment, we would like to indicate that clonogenicity is considered a measure of cell proliferation and, because of this is an in vitro indicator of
tumorigenicity. As examples for reference, we list Reya et al. 2001 and Wicha et al., 2006. However and as indicated by the referee, it does not automatically exclude the possible involvement of additional mechanisms.


3.- From our neurosphere assay, we concluded that Msi1 may have a role in sustaining cancer cells. We use the words “sustain” and “maintain” to actually avoid making the statement that the differences observed in our Neurosphere assay were due merely to a reduction in cell proliferation therefore leaving open the possible participation of additional mechanisms. Following the referee’s suggestion, data has been included in figure 3 (panel 3.c) showing the effect on overall neurosphere size after Msi1 depletion. The sentence on page 10 has been also changed to more accurately describe our results. Where it said “As shown in Figure 3, on average a 2-fold reduction in both number and overall size of the spheres….” now it says: “As shown in Figure 3, a significant reduction in both number and overall size of the spheres …..”.

4.- As indicated in point #1, we do not conclude that “upregulation of Msi1 in neurospheres reflects its role in cancer progression” but rather that the high levels of Msi1 in neurospheres (actively proliferating cultures) versus monolayers (less actively proliferating cultures) “suggested a potential role for Msi1 in promoting cancer cell proliferation in the medulloblastoma cell line Daoy”.

We decided to analyze gene expression in monolayer culture rather than in neurospheres as cells expressing high or low Msi1 levels exhibited similar growth rates. Indeed, we believe that the use of neurosphere cultures is not the best way to test for differential expression of cell proliferation genes. The neurosphere assay already implies a selective pressure. As only the cells “less impaired” inside the Msi1 KD population are the ones able to form neurospheres, very likely, the differences in gene expression between both cell lines will become diluted.

We have now incorporated the sequence targeted by the shRNA on page 4. For clarification purposes; we did check the gene expression profiles of the three isolated clones and they were very similar.
5.- We’ve quantified the differences in neurosphere size after cyclopamine treatment in control and KD cells. These new data is included in figure 5 (panel 5.b). We’ve scored the number of spheres larger than 100µm in diameter in 10 different fields. In the presence of tomatidine, the number of neurospheres was significantly smaller in KD cells compared to control Daoy cells (p< $10^{-4}$). The effects of cyclopamine treatment upon neurosphere formation were more severe in KD cells as significant differences were observed when cells were treated with 5 µM cyclopamine (p< 0.05) while 20 µM cyclopamine was necessary to observe significant differences (p<0.01).

We appreciate the referee’s concerns and therefore we have incorporated different field images in figure 5 to better illustrate the effects of cyclopamine upon neurosphere formation in Msi1 KD cells.

As indicated by the first referee, a plausible conclusion from this experiment is that Hedgehog may play an essential role in Daoy neurosphere formation and the enhanced sensitivity to cyclopamine detected in the Msi1 KD might be due to a partial repression of this signaling pathway in this cell line. This clarification has been now incorporated in the manuscript.

6.- We agree with the referee that to prove apoptosis a specific assay needs to be performed. We presented the Bcl-2 data, as it was suggestive of the potential involvement for Msi1 in cancer cell’s apoptosis. However, and although intriguing, this is beyond the scope of the present manuscript.

Yours truly,

Luiz O. Penalva