Author's response to reviews

Title: Clock gene Bmal1 is dispensable for intrinsic properties of murine hematopoietic stem cells

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Author's response to reviews: see over
Dear Dr. Reinhardt,

We would like to express our gratitude to the reviewers for their detailed comments and suggestions to improve the quality of our manuscript. Please find enclosed revised version of manuscript entitled “Clock gene Bmal1 is dispensable for intrinsic properties of murine hematopoietic stem cells” by Ieyasu et al.

We have changed some sentences and performed the following revisions in line with the suggestions made as listed in the point-by-point reply below.

We very much hope that these changes are satisfactory to yourself and the reviewers, and that our manuscript may now be acceptable for publication in JNRBM.

Yours sincerely,

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Reviewer number: 1

Reviewer's report:

Minor Essential Revisions

Prior studies have reported that mobilization of hematopoietic stem and progenitor cells (HSPCs) and neutrophils from bone marrow into the circulation is regulated by circadian oscillations (Mendez-Ferrer et al., Nature 2008; Casanova-Acebes, M et al., Cell 2013). The authors tried to determine if HSC behavior is regulated by an essential component of the master circadian pacemaker, Bmal1, using Bmal1 deficient mice. They demonstrated that the numbers, functions and cell cycle progression of HSCs were unaffected in the mutants. The subject matter is of interest and potential importance.

Minor points

The authors should include the data shown in Supplemental Figure 1A indicating that circadian fluctuations of circulating CFU-Cs between ZT5 and ZT17 were affected in Bmal1 deficient mice in the manuscript.

We appreciate the reviewer’s advice. For better understanding of experimental results, we have changed the sentences in page9, line 7-13 as follows.

“Indeed we could detect oscillating CFU-Cs of HSPCs in PB of Bmal1<sup>+/+</sup> mice at Zeitgeber time (ZT) 5 and ZT17, but there were no statistically significant fluctuations in case of Bmal1<sup>−/−</sup> mice (Supplemental Figure 1A). Thus, oscillating CFU-Cs of HSPCs appear to be regulated by circadian clock, however, it is unclear how Bmal1 affects intrinsic functions of HSCs such as differentiation, proliferation and repopulating capacity. We therefore asked to investigate and clarify these problems.”
Reviewer number: 2

Reviewer's report:
The subject underlying this paper is very interesting as it aims to determine the relevance of circadian clock gene bmal1 regarding properties of HSC. Even though data presented suggests Bmal1 is dispensable, the authors fail to clearly demonstrate it.

Major Compulsory Revisions
1 - The paper is poorly and carelessly written, and it refers to figures which are not present in the paper. Both in figs 1 and 2 legends describe a) to e) although it only presents a) to d). Figures indications through the text are not always correct making it too difficult to follow.

We apologize for causing confusion. We have added Figure 1E-F and 2E. To explain our results more clearly, some sentences have been corrected accordingly.

2 - In page 9 the authors state that there are no differences in Figure1b however I find the cells in panel Bmal1+/+ smaller than Bmal1-/- but cannot be sure of these differences as there is no scale bar in the figures or indication of the magnification in use in the legend.

We acknowledge the reviewer’s concerns. We have replaced Figure 1B with a new one having a scale bar, and indicated the extent of magnification in the legend. After close examination, we came to the conclusion that there is no significant morphological difference between the colonies of two groups.

Furthermore authors say that “Bmal1+/+ and Bmal1-/- CD34-KSL cells demonstrated comparable proliferation potentials” but the figure is missing.

We thank the reviewer’s pointing out our mistake and once again our apologies for the confusion. We have add figure 1C, showing comparable proliferation potentials of Bmal1+/+ and Bmal1-/- CD34 KSL cells.

3 - In page 10 the authors affirm that “4, 8 and 12 weeks after transplantation, (…) Bmal1+/+ and Bmal1-/- BM cells were equally capable of hematopoietic reconstitution” but only show results from 12 weeks. The same happens with the second BMT data. I would like to see 4 and 8 weeks data. Still regarding this data I would like to see the
statistical analysis, the figures suggest that may be less granulocytes and macrophages in both Bmal-/- first and second BMT, more B cells in the first Bmal-/- BMT, and more CD3+ T cells in the second BMT, although I do not know whether it is statistically significant.

In accordance with the reviewer’s comments, we have added 4 and 8 weeks chimerism data in supplemental figure 1B (1st BMT) and 1C (2nd BMT).

At 4, 8 and 12 weeks after transplantation, flow cytometric analysis showed a high-level chimerism of B220+ cells in PB of the recipients transplanted with Bmal1+/+ BM cells, but this was not observed in the second BMT. In addition, there was no statistically significant difference in the chimerism of Gr-1+/Mac-1+ and CD3+ cells, suggesting that overall BM reconstitution ability was not altered due to Bmal1 deficiency.

**Minor Essential Revisions**

4 - Regarding circadian oscillations, even though CFU-C's have a high peak at ZT5 and low at ZT17, it would be enriching to have a point at ZT11 in CD34-KSL analysis to be sure that it does not oscillate. It could oscillate off-phase of CFU-C's

We compared the relative frequencies of Bmal1+/+ and Bmal1-/- CD34+KSL cells at ZT5, 11, 17 and ZT23, and found that there is no statistically significant difference between two groups.

**Figure.** The frequency of HSCs in the BM of 8-10-week-old Bmal1-/- mice at ZT5, 11, 17 and 23. The mean percentage ± SDs of CD34+KSL cells at ZT5 (n = 4) and ZT11, 17 and 23 (n = 3)