Format for ANSWERING REVIEWERS

3rd of December 2013

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name wjh-2012-000011-review .doc).

Title: *Ex vivo* expansion of hematopoietic stem and progenitor cells: recent advances

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Manuscript No: wjh-2012-000011

The manuscript has been improved according to the suggestions of reviewers:
1. Format has been updated
2. Revision has been made according to the suggestions of the reviewer

We added 1 additional author to the revised manuscript because of significant contribution. We hope that our significant revision improved the manuscript to satisfactory level. As we mentioned on at first submission, we would like to request following investigators to exclude from the reviewing process due to potentially competitive nature, although we should emphasize that they are outstanding scientists.

- Dr. Hal E. Broxmeyer (Indiana University)
- Dr. Irving L. Weissman (Stanford University)
- Dr. Shahin Rafii (Cornell University)

If you have any questions, please contact the corresponding author, Katsuhiro Kita (kakitatemp@yahoo.com or kak2040@med.cornell.edu).

Reviewer #1

(1) Thank you for the very positive comment on our manuscript. Although we agree that it is increasingly important to discuss the same line of studies in the other stem cells, such as iPS and embryonic stem cells, we think that it is going to be overwhelming in *Editorial* review (relatively short format, too) and it is beyond the scope of this manuscript as already stated on the title. Therefore, we hope that the reviewer understands the focus of the manuscript and thus we omit to expand the discussion about iPS and hESC.

Reviewer #2

(1) As you suggested, there is one recent study that subsequently tested the effect of aryl carbon receptor antagonists in a feedback culture system. This paper is cited as an additional study using aryl hydrocarbon receptor antagonists in HSC expansion (ref. 43). Although one most recent study (B.W. Smith *et al.* (2013) *Blood*, **122**, 376-385) described the role of aryl hydrocarbon receptor signaling in hematopoietic progenitor's fate, the study basically showed megakaryocyte- and erythroid-lineages differentiation of progenitor cells upon aryl hydrocarbon receptor activation. Thus, we did not cite the latter paper.

We also significantly expanded discussion, and made one sub-section (iv) small molecule (pages 13~14), where copper chelator and the other chemicals are discussed. Hope these changes improve the manuscript.

(2) Thank you for pointing out lacking information. We agree that we have to discuss the other growth factors/cytokines as well, since our previous version did not cover enough. G-CSF and TPO are the other very important cytokines missing here. As first introduced by Shpall and colleagues, G-CSF/TPO/SCF was one of the cytokine cocktails tried in early studies. We should also discuss endothelium-derived factors as suggested in your comment. We overhauled this part and you can find the detailed discussion in pages 9~13 (subsections 1. soluble factors, 2. developmental regulators, 3. stromal

support (fibronectin)). We hope this part is improved.

(3) Thank you so much for your opinion. We cited recent original and review articles that you suggested, and ed the manuscript to expand discussion as mentioned above. Since we still would like to keep the original title intact (although we left "recent advances", we still felt that amendment of the title is better and changed the title to "…hematopoietic stem *and progenitor* cells…". This is also because the other reviewer pointed out the purity of hematopoietic stem cells that are discussed in this review, and we think that our discussion includes progenitor cells as well. Also thank you so much for reminding us additional articles to be cited. Review articles (W. Hai-Jiang *et al.* (2008) *Am. J. Hematol.*, **83**, 922-926 & A. Dahlberg *et al.* (2011) **117**, 6083-6090) are cited in introduction (refs. 6, 7), and angiopoietin-like 5 and IGFBP2 paper is cited in soluble factor sub-section under "ex vivo culture media for HSC expansion" (page 11, lines 3~5; ref. 71).

Reviewer #3

(1) Thank you for pointing out unclear part in the manuscript. As mentioned, it is true that markers discussed here are still not enough to identify the most primitive HSCs. Therefore, first, we amended the title as "*Ex vivo* expansion of hematopoietic stem and progenitor cells: recent advances". Second, we should clearly state that the fraction we are discussing here are actually hematopoietic stem and progenitor cells. We stated this clearly at page 3 bottom (lines 18~21) "[Hereafter we use the term HSCs to represent both HSCs and hematopoietic progenitor cells...the term "HSCs" used throughout this review includes the $CD34^+$ fraction.]"

The second part of this question – about human and mouse HSCs (or may include progenitor cells) – we mention that one of the markers that are often combined with the other molecules, Sca-1, is only expressed in mouse (page 5, line 12). We also extensively expanded our discussion related to CD34 expression including a possible technical pitfall (page 5, line 14~page 6, line 21). We think most of published review articles do not discuss CD34 issues like this section, and we hope that our discussion is one-of-a-kind and stands out. In terms of most primitive hematopoietic stem cells, recent paper by John Dick's group may be one of the appropriate papers describing the combination of cell surface antigens to obtain the highest purity of hematopoietic stem cells, hence, we mentioned on the paper (page 5, lines 26~29; "*Most recently, John Dick's group isolated…can generate the entire hematopoietic stem cells*.").

(2) This is a good question. As you pointed out, influx of Hoechst dye can also be used to select sub-population of HSCs. Pearce and Bonnet compared the phenotypes of cells selected by either ALDH and Hoechst dye efflux (Exp. Hematol. (2007) 35, 1437-1446). Interestingly, Hoechst side population only contains $CD34^+$ cells. Therefore, although Hoechst dye can be used to roughly select HSC-enriched fractions, it appears not to be suitable to enrich certain sub-population, such as $CD34^-CD38^+$ cells. Rhodamine 123-based staining was also reported for a long time ago, however, it may not be suitable because of…. In addition, although it may not be strong enough to consider, cytotoxicity of Hoechst 33342 was reported for a long time ago (R.E. Durand & P.L. Olive (1982) J. Histochem. Cytochem., 30, 111-116; A.Y. Chen *et al.* (1993) *Cancer Res.*, 53, 1332-1337). Supporting these pioneering studies, Hoechst 33342 inhibition of erythroid progenitor clonogenecity was reported (B. Machalinski *et al.* (1998) *Ann. Transplant.*, 3, 5-13) Therefore, we think that ALDH activity-based selection may be the best available method to enrich HSCs. All these issues are discussed details (page 7, lines 4~20; "*Prior to this method,…ALDH-based selection may be the best option to sort/enrich HSCs*.").

(3) Thank you for the comments. We re-structured the descriptions of cytokines on pages 9~10. In addition to the routes of them, we developed the routes of each cytokine/growth factor (page 9 (i) soluble factor~page 14 (iv) small molecules). Regarding the importance of serum-free culture systems, we mentioned on the reasons at the beginning of the section, "Ex Vivo culture media for HSC expansion" on page 8, line 23~ page 9, line 1; "*Development of serum-free culture is…to allow significant expansion of enriched HSCs.*").

(4) We agree with you that HSC exhaustion is an important topic to be discussed in this review. In our understanding, the loss of long-term hematopoiesis in dividing HSCs is mainly because of exhaustion of HSCs. It is mention on page 9, lines 3~19; "*Another major hurdle is current…*" and the discussion including knockout mice studies is developed on page 14~16 (A new section "Expansion versus *in vivo* reconstitution"). We hope these major changes are sufficient to answer to the question.

3. References and typesetting were corrected

Thank you again for an opportunity to publish our manuscript in the *World Journal of Hematology.*

Sincerely yours,

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