INDIANA UNIVERSITY

DEPARTMENT OF PEDIATRICS
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Dear Editors-in-Chief of World Journal of Stem Cells,

On behalf of my co-authors, we are pleased to submit a revised version of our invited manuscript (#55027) entitled “How Old is Too Old? In vivo engraftment of human peripheral blood stem cells cryopreserved for up to 18 years - implications for clinical transplantation and stability programs”. We want to thank the reviewers for their suggestions, and we made all of the changes recommended by the reviewers (see Response to Reviewers below).

Please find in our revised submission all the documentation required by the Journal, as described in the April 2, 2020 Notification email. Again, please note that the Institutional Review Board and Animal Care approvals are from 2012 and 2011, respectively. These dates are correct; the experiments were performed and presented in abstract form in 2013 but have not been written in complete manuscript form until now. Please note that we have also updated the title to say “up to 18 years”; this change was made for consistency and clarity, since the mean cryopreservation time of the PBSC units studied was 17 years, but the oldest units were over 18 years old.

Of note, we have made the changes indicated by the Editor according to the “Further Revision Needed” email sent yesterday, 4/12/2020. Specifically, we have

1. **Included documentation of the funding used to perform this work;**
2. **Added the Article Highlights at the end of the main text;**
3. **Updated the manuscript including the author contributions and footnotes – the revised manuscript now looks exactly like the “Format for Manuscript Revision Basic Study” example given on the website.**

Thank you again for inviting us to submit this manuscript; we hope the revised manuscript satisfies the reviewers and is now acceptable for publication. Please do not hesitate to contact me if I can provide any additional information useful for the review process.

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Best regards,

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Response to Reviewers:

03471230 **Conclusion:** Minor revision
Scientific Quality: Grade C (Good)
Language Quality: Grade B (Minor language polishing)
The capacity of successfully store and thaw long-term cryopreserved products maybe is a key step for some autologous patients. In this study, the authors reported that PBSC could engraft after cryopreservation for mean of 17 years. The results showed that BFU-E growth was shown in 9 of 10 cryopreservation (range 13.6-18.3 years) PBSC units and CFU-GM growth in 7 of 10 units post-thaw. Immunodeficient mice transplanted with CD34+cells, picking randomly from the cryopreservation PBSC, and demonstrated with the presence of 34±24% human CD45+ cells and its differentiation at 12 weeks. The growth of erythroid and myeloid colonies was also found in the harvested bone marrow from all mice. But, I also have some comments: 1.There are several stem cells sources with great properties, such as umbilical cord blood stem cells and bone marrow mesenchymal stem cells, as significant candidates for stem cell transplantation in clinic treatment. Is it essential to investigate the properties of long-term cryopreserved PBSC in clinical trials? 2.A new life hematopoiesis function of immunodeficient patient. It needs more results to support the engraftment results in the report.

Response:

1. The authors thank you for your comments. We studied PBSC because A) PBSC are by far the most used source of HSC; B) PBSC are often frozen prior to transplantation; and C) while there is published data on long-term cryopreserved bone marrow and umbilical cord blood, there are no reports of the utility of long-term cryopreserved PBSC. Previous studies of cryopreserved PBSC are limited to 11 years or less; our study establishes that PBSC units frozen up to 18 years can still engraft. Thus, our investigation fills a gap in the existing literature. Information about the PBSC usage and the supporting references are found in the Introduction, pages 4-5 of the manuscript.
2. We think the reviewer is asking here why we didn't transplant more PBSC units into mice. In this study, we were simply seeking to demonstrate that a representative sample from the 10 long-term cryopreserved PBSC units could engraft in immunodeficient mice. This was not intended to be a comprehensive study of PBSC engraftment in NSG mice, since PBSC engraftment in mouse models is already well established. We had funding to purchase and transplant about 25 NSG mice, and therefore chose to transplant PBSC from four thawed units into each of 6-7 recipients. In addition, other published studies have transplanted similar numbers of cryopreserved HSC units to validate engraftment in immunodeficient mice (references 22-24) or only in vitro (reference 18). A sentence in the Methods (page 8) has been clarified, stating that we transplanted four randomly chosen PBSC units as a representative sample from the 10 units to investigate if the units could engraft in immunodeficient mice.

Furthermore, we showed that transplantation of long-term cryopreserved PBSC units into immunodeficient mice results in long-term engraftment with reconstitution of a full immunophenotype of human cells (Results, page 10 and Table 2). Functional immune reconstitution, however, can only be demonstrated in human transplants, not mouse models.

02726701**Conclusion:** Minor revision
Scientific Quality: Grade B (Very good)
Language Quality: Grade A (Priority publishing)
Comments on How Old is Too Old? In vivo engraftment of human peripheral blood stem cells cryopreserved for up to 17 years - implications for clinical transplantation and stability programs Introduction Clear and well written. Material and Methods Please, add a brief description of the freezing and thawing protocols Results It is not clear, why did authors randomly select 4 of 10 PBSC units and did not test all units (Tables 1 and 2). At the same time, was it a coincidence that the four randomly selected PBSC units came from the first four available (A to D)? Please, clarify. Discussion A little bit long, but adequate. Tables: Commented. Abstract and Core tip: OK. In summary. The manuscript needs minor edition to be suitable to be published.

Response:
1. The authors thank you for your suggestions. Additional details of the freezing and thawing process have been added to the Methods section (pages 6-7).
2. Please see our response to the other reviewer above. In this study, we wanted to demonstrate that a representative sample from the 10 long-term cryopreserved PBSC units could engraft in immunodeficient mice. We chose four PBSC units for

transplantation both due to funding, and because other investigators used similar numbers of transplanted mice to prove that long-term cryopreserved cord blood units could engraft (references 22-24).

3. The units for transplantation were chosen randomly; we simply labeled the transplanted units A-D and the in vitro-analyzed units E-J when making Table 2. As noted above, we clarified a sentence (Results, page 8) stating that four randomly chosen PBSC units were transplanted as a representative sample from the 10 units to demonstrate that they could engraft in immunodeficient mice.