[11/03/2016]

Ze-Mao Gong
Science editor
World Journal of Gastroenterology

Dear Dr. Ze-Mao Gong,

We are submitting our revised version of manuscript **(ESPS Manuscript NO. 30036)** "Long-term culture-induced phenotypic difference and efficient cryopreservation of small intestinal organoids by optimal treatment timing of Rho kinase inhibitor" by Sung-Hoon Han, Sehwan Shim, Min-Jung Kim, Hye-Yun Shin, Won-Suk Jang, Sun-Joo Lee, Young-Woo Jin, Seung-Sook Lee, Seung Bum Lee and Sunhoo Park for publication in *World Journal of Gastroenterology* as an original research article.

We thank the Reviewer and Editors for the insightful comments on our manuscript. We have carefully addressed all the points they have raised. The text in the manuscript has been revised in line with the reviewer suggestion.

Detailed responses to Reviewer and Editors comments are provided in attached pages. All changes in the manuscript are highlighted by blue colored words. We also updated the all required documents (No. 1~14).

We would like to extend our thanks to the positive and critical comments of the reviewers and to you for a chance to resubmit our manuscript.

I hope that we satisfactory addressed yours and reviewers concerns and the revised manuscript is now acceptable for publication in *World Journal of Gastroenterology*

Sincerely,

Sunhoo Park, M.D./Ph.D.
Laboratory of Radiation Exposure & Therapeutics
National Radiation Emergency Medical Center
Korea Institute of Radiological and Medical Science
75 Nowon-ro, Nowon-gu, Seoul, Republic of Korea
Telephone: +82-2-970-1274
Fax: +82-2-970-2430
E-mail: sunhoo@kirams.re.kr

Response Letter

ESPS Manuscript NO: 30036

Manuscript Title: Long-term culture-induced phenotypic difference and efficient cryopreservation of small intestinal organoids by treatment timing of Rho kinase inhibitor

Authors List: Sung-Hoon Han, Sehwan Shim, Min-Jung Kim, Hye-Yun Shin, Won-Suk Jang, Sun-Joo Lee, Young-Woo Jin, Seung-Sook Lee, Seung Bum Lee, Sunhoo Park

Step 1. Please revise your manuscript according to the reviewers' comments

We appreciate the Reviewer suggestion and revised the manuscript. As suggested, all changes were highlighted in the updated version of the manuscript by colored word. Please find changes in detail below.

Reviewer(s)' Comments to Author:

The manuscript by Han et al described that phenotypes of mouse intestinal organoids under ENR media were maintained over a long duration, and organoids under ENR-CV media exhibited morphological alterations. They also found that adding the Rock inhibitor Y-27632 during freezing benefits recovery of undissociated intestinal organoids after long-term cryopreservation. The manuscript is succinct and the conclusions are well supported by the data. However, the authors should consider the following comments to improve the manuscript. Specific comments are as follows:

Major comments:

1) The author used same methods as the previous paper of Grabinger et al (2014, Cell Death and Disease). However, based on our knowledge, Matrigel may affect the contact of particular chemicals/growth factors with organoids to some extent. Herein, I suggest that the authors could verify MTT results under condition that organoids can be cultured without Matrigel (for instance, with Collagen R coated plate).

We appreciate of the insightful comments. As reviewer suggested, we performed the experiment of culturing crypts on collagen-coated plate. As shown below (please refer to the attached Figure), these crypts could not be expanded and were going to be dead as observed by morphology and MTT analysis. This result is consistent with previous reports (Grossmann J et al., 1998, The American journal of pathology, Ref no. 6; Kaeffer B, 2002, In

vitro cellular &developmental biology Animal, Ref no. 7 in manuscript) that primary intestinal crypts are difficult to expand in monolayer culture due to rapid cell death as described in page 5.

For the first time, using a Matrigel matrix-based on 3D culture system, organoid culture representing the recapitulation of crypt-villus *in vivo* architecture was firstly established by Sato T et al (2009, Nature, Ref no. 8). Since that report, most groups have been used to Sato protocols for organoid culture including paper of Grabinger et al (2014, Cell Death and Disease). Most people have been demonstrated their functional study by using various pharmacological inhibitors/growth factors under matrigel-based on 3D culture condition. We also established organoid culture system showing recapitulation of the characteristic crypt-villus architecture by adapting method of Sato T et al. Thereby, we agree that the effect of Matrigel regarding to the contact of particular chemicals/growth factors with organoids to some extent is considered, but feel that it is beyond the scope of this investigation.

Figure for reviewers' comment

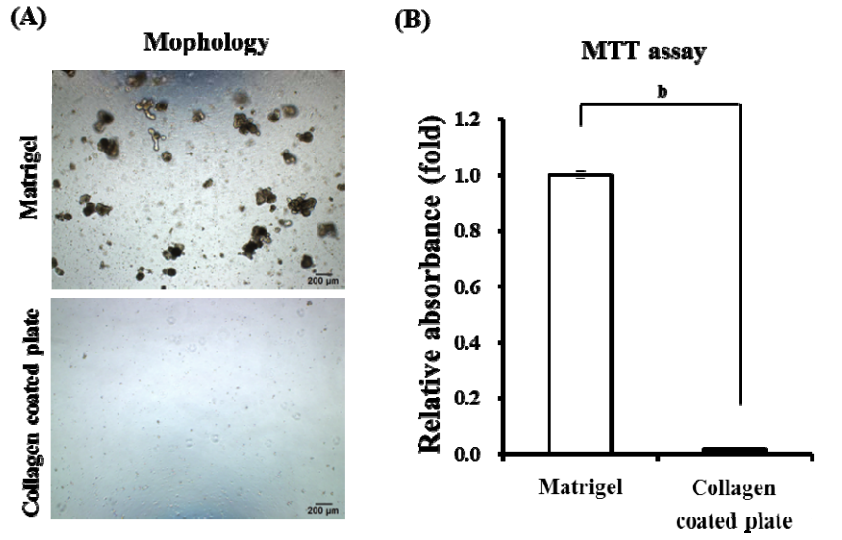


Figure for Reviewer comment. Isolated crypts were resuspended in either Matrigel (3D) or Collagen-coated plate. (A) Representative morphology and (B) MTT assay of organoids cultured on day 7 under ENR media.

Addition comments:

1) In MATERIALS AND METHODS, usually, P value of less than 0.05 is considered to be significant, why authors mentioned 0.01?

We agree with Reviewer suggestion and corrected it as 0.05 in manuscript in page 10.

2) In Fig 1A, the authors stated that under ENR-CV conditions yielded increased budding length and size compared with those of organoids cultured under ENR conditions. Was this phenomenon was observed in a few organoids or most of the organoids?

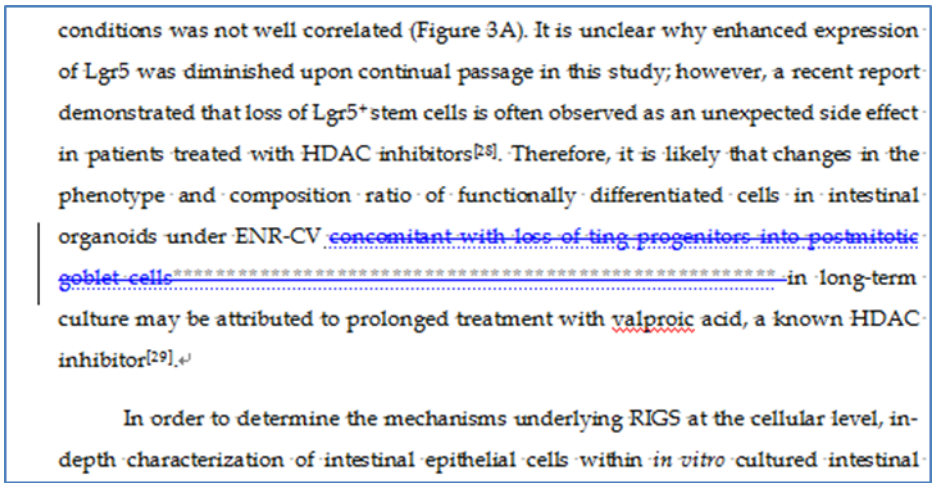
We observed this phenomenon from most of the organoids which is consistent with previous report (Yin X et al., 2014, Nature methods, Ref no. 9 in manuscript).

3) Explain why the morphology of ENR-based organoids was constantly maintained; whereas the enhanced size and budding length of organoids in ENR-CV culture conditions were gradually diminished after passage 8 (P8) (Figure 2A)?

We thank the reviewer for raising this issue. The only different thing between ENR and ENR-CV is inclusion of small molecules "CHIR99021 and Valproic acid" indicating C and V. Since each small molecule was known to activate Wnt and Notch pathway respectively, we thought that interrupt of continually activated-Wnt or Notch signaling might be associated with these events. However, we could not have any clues from Real-time PCR analysis. The reasons why the phenotype of intestine organoid under ENR-CV condition upon extended passage was changed, are not clear at present. According to a previous report (Zimberlin et al., 2015, FASEB J, Ref no. 28 in manuscript), we assumed that these change may result from prolonged treatment with valproic acid, HDAC inhibitor which gives rise to unexpected side effect such as loss of intestinal homeostasis. **The detailed explaining was described in Discussion (page 15~16).**

4) In DISCUSSION, there are some strange characters (page 16), please modify it.

We apologize for these strange characters in manuscript. This event seems to be due to different version of MS word. The strange characters including some words (page 16) as shown in attached screenshot were deleted in manuscript.



conditions was not well correlated (Figure 3A). It is unclear why enhanced expression of Lgr5 was diminished upon continual passage in this study; however, a recent report demonstrated that loss of Lgr5^{+} stem cells is often observed as an unexpected side effect in patients treated with HDAC inhibitors[28]. Therefore, it is likely that changes in the phenotype and composition ratio of functionally differentiated cells in intestinal organoids under ENR-CV ~~concomitant with loss of ting progenitors into postmitotic goblet cells~~ in long-term culture may be attributed to prolonged treatment with valproic acid, a known HDAC inhibitor[29].

In order to determine the mechanisms underlying RIGS at the cellular level, in-depth characterization of intestinal epithelial cells within *in vitro* cultured intestinal

5) In the manuscript, some date are presented as SEMs, while some are presented SD. Is there a specific reason? Otherwise, this should be consistent.

We appreciate of your comment. We used SEM to present the comparing independent groups which were performed experiments with each passage during long-term culture (more than 3 months), followed by classifying early phase (P0-4) and late phase (P8-12) as shown in Figure 2 and 3.

Editor(s)' Comments to Author:

We appreciate the editor's suggestion and revised the manuscript. As suggested, all changes were highlighted in the updated version of the manuscript by colored word. Please find changes in detail below:

Memo 1) Submit the scientific research process

We uploaded above-mentioned document during revision process.

Memo 2) Please revise and perfect your manuscript according to peer-reviewers' comments

We revised our manuscript according to reviewers' comments.

Memo 3) Add postcode here

We added postcode (01812) in revised version in page 1.

Memo 4) You need to provide the grant application form(s) or certificate of funding agency for every grant, or we will delete the part of "Supported by...".

The approved grant application forms are uploaded with the revision of this manuscript.

Memo 5) Just one author here

We changed it in revised version as you suggested.

Memo 6) Please check that there are no repeated feferences

There is no repeated references when we check it again.

Memo 7) Please provide the decomposable figure of Figures, whose parts are movable and editable. So please put the orginal picture as PPT so that we can edit them easily.

We put the original pictures (Figure 1) as PPT in revised version as you suggested.

Memo 8) Please provide the decomposable figure of Figures, whose parts are movable and editable. So please put the orginal picture as PPT so that we can edit them easily.

We put the original pictures (Figure 2) as PPT in revised version as you suggested.

Memo 9~13) Change it to “b”

We changed it in revised version as you suggested.