Sep 30, 2019

Dr. Ying Dou
Editor in Chief
World Journal of Stem Cells

Dear Dr. Ying Dou,

We would like to thank you for reviewing our manuscript, titled “Inducing human iPSC differentiation through embryoid bodies: A practical and stable approach.” (47987). We would also like to thank the reviewers for their time and comments in evaluating this manuscript.

In accordance with the suggestions from the editors and the reviewers, we have adjusted the whole structure of the manuscript and some writing formats. We highlight the revised content as shown in “47987-manuscript（Edicted Revised）-file”. Meanwhile, we have carefully checked and confirmed that there are no repeated references. We have also responded to the reviewers’ comments point by point (please see “Answering reviewers”) and revised the manuscript accordingly. We would be glad to answer any further questions and comments that you may have.

Best regards,

Yun-Wen Zheng
University of Tsukuba
Department of Advanced Gastroenterological Surgical Science and Technology
1-1-1 Tennodai
Tsukuba, Ibaraki 305-8577
JAPAN

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Point-by point Responses to the reviewer’s comments

Responses to the Reviewer 1:

This review article summarizes studies on formation of embryoid bodies as a method

to determine iPSC differentiation. While the topic is very interesting and important, this reviewer found the article hard to follow. This is not due to the Language, that is quite good, needing only minor polishing, but to the general organization of the text that is unclear. This manuscript could be considered for scientific publication after a major revision.

Thank you very much for your kind reviewing and comments. We really understand and accept what you pointed out. According to your comments, we have made a major revision with logical re-organization. Firstly, we summarized the current wide application of embryoid body (EB)-mediated iPSC differentiation and their great advantages especially in scaling up culturing, differentiation efficiency enhancement, *ex vivo* simulation, and organoid establishment. Secondly, to improve the stability and feasibility of EB-mediated differentiation and generate high quality EBs, we analyzed and discussed some critical factors involved in EBs generation on the basis of both published data and our own laboratory experiences, including iPSC pluripotency maintenance, generation of uniform morphology using micro-pattern 3D culture systems, proper cellular density inoculation, and EB size control. We hope the revised manuscript will be easy to follow.

Question 1:- to provide early in the manuscript a clear definition of embryoid body, both in the abstract and introduction.

Response:
We are sorry that we missed that. We have already provided a clear definition of embryoid body, both in the abstract and introduction of the revised manuscript. The detailed contents are as follows:

In abstract - *Embryoid bodies (EBs), the multicellular aggregates spontaneously generated from iPSCs in the suspension system, might help to address these issues.*

In introduction - *EB is a multicellular aggregate spontaneously formed by pluripotent stem cells under suspension culture conditions, which has three germ layer structures and partially recapitulate the early embryonic development[1]. Such a multicellular 3D structure improves cell-cell contacts and intercellular communication and also enhances substance exchange[2]*

Question 2:- at the end of the introduction, a short paragraph on the organization and summarized content of the following paragraphs of the review should be provided.

Response:

Thanks for the practical suggestion and we have added the summary at the end of introduction as follows:

In order to understand the critical events of EB-mediated differentiation, explore better methods and solve the aforementioned problems, we recapitulated the current applications and advantages of using EBs in iPSC differentiation. Combining our own and previously published data related to EB formation and differentiation, we conducted a comparative and predictive analysis and aimed to provide a reference to create a more stable and practical way of high-quality EB generation

Question 3:-in the introduction, the authors state that "In this review, we conducted an analogical analysis of the current status of iPSC-derived differentiation, especially through EB formation, in an attempt to explore an effective system combining the key influencing factors throughout the differentiation period, and to shorten the culture cycles as far as possible". Analogical analysis in an inusual term in this context: what do the authors mean? Could the authors provide further details?

Response:

We are so sorry that this term make you confused. We used “comparative and predictive analyses” in the revised version and we hope it is more appropriate.

Question 4:-The sentence: "It is worth noting that an aggregate is defined as an EB if it is formed in the absence of inducing factors" does not fit well as the conclusive sentence of the introduction. It may be moved earlier in the introduction, when the definition of EB is provided.

Response:

We have provided a clear definition of EB and deleted this sentence in the revised manuscript.

Question5:-In the section "2.1 Pluripotency maintenance, passaging, and aggregation" the authors use several acronyms without providing a definition: each acronym used should be clearly defined (e.g., ROCK, GSK, MEK, bFGF) throughout the text.

Response:

Thank you for your suggestions and we have provided the full name for each acronym.

Question 6:-The sentence "they suggested that bFGF with three other inhibitors occasionally constitutes a culture environment that is more easily adaptable", implies

that bFGF is an inhibitor of what? What are the other inihibitors tested in this study? These details should be added to the text.

Response:
bFGF is not an inhibitor and we are so sorry that our description leads to misunderstanding. In the study of Tsutsui H et al[3], they used a feedback system control scheme to optimize an efficient combination and concentration of small molecules which can support long-term maintenance (>20 passages) of hESC culture through routine single cell passaging, without serum and feeders. It is demonstrated that the combination of bFGF and three inhibitors of ROCK, GSK, and MEK is crucial for the maintenance of hESCs. However, bFGF alone or combination of bFGF with other small molecule is insufficient for hESCs in long-term culture. Therefore, bFGF is crucial for hESCs stemness maintenance and it shows particular role in single cell passaging together with the inhibitors of ROCK, GSK, and MEK.

Question 7: The last paragraph of section 2.2 and Figure 1 seem to contain original data from the authors and contain no reference to previous publication and they should be removed, at this is a review article, unless the authors can add a reference (even to a conference proceeding or similar content).

Response:
Thank you very much for your comments. In this revised manuscript, we combined the published data and our own laboratory experiences (including Figure 1 and other data such as cellular density and EB size) relating to the quality control of EB and we also conducted a comparative and predictive analysis. We hope these results will be helpful to the improvement of EB formation in the future.

Question 8: To this reviewer seems that section 4 would be more useful to the reader, to follow the review article flow, if moved earlier in the text perhaps after the introduction?

Response:
We have revised the structure of the whole manuscript and moved this section after the introduction (1. Application and advantages of EB use in iPSC differentiation) as you advised.

Question 9: A conclusive sentence is missing in the text.

Response:
A conclusive sentence have been provided at the end of the text, the detail as follows：
In conclusion, the production of a large quantity of homogeneous EBs with high

quality is important for many PSCs related studies, including scaling up culturing, organoid formation, and differentiation potential prediction.

References

1 **Kurosawa H**, Methods for inducing embryoid body formation: in vitro differentiation system of embryonic stem cells. *Journal of Bioscience & Bioengineering*. 2007; **103**(5):389-398 [PMID: 17609152 DOI: 10.1263/jbb.103.389].

2 **Saltzman WM** and Kyriakides TR, Chapter 20 - Cell Interactions with Polymers, in Principles of Tissue Engineering (Fourth Edition), R. Lanza, R. Langer, and J. Vacanti, Editors. 2014, Academic Press: Boston385-406 [Doi: 10.1016/B978-0-12-398358-9.00020-3].

3 **Tsutsui H**, Valamehr B, Hindoyan A, Qiao R, Ding X, Guo S, Witte ON, Liu X, Ho C-M, and Wu H, An optimized small molecule inhibitor cocktail supports long-term maintenance of human embryonic stem cells. *Nature Communications*. 2011; **2:**167 [PMID: 21266967 DOI: 10.1038/ncomms1165].