Format for ANSWERING REVIEWERS

May 19, 2015

Dear Editor,

Thank you for reviewing our manuscript. We greatly appreciate the helpful comments from the reviewers. We have revised the manuscript incorporating/addressing the review comments as well as providing new data as suggested by the reviewers.

Please find enclosed the edited manuscript in Word format (file name: 17168-review.doc).

Title: Simplified three-dimensional culture system for long-term expansion of embryonic stem cells

Authors: Christina McKee, Mick Perez-Cruet, Ferman Chavez, G. Rasul Chaudhry

Name of Journal: *World Journal of Stem Cells*

ESPS Manuscript NO: 17168

The manuscript has been formatted as advised:

1. All necessary documents, including the Answering Reviewers response letter and Copyright Assignment form, have been signed and uploaded.

2. An audio file describing the final core tip has been included.

3. This research is conducted with cell lines purchased from ATCC and has been approved by the Oakland University Institutional Biosafety Committee (IBC protocol number: 1814). All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Oakland University (IACUC protocol number: 14033). Institutional review board statement and informed consent statement are not applicable since the study did not involve human subject research.

4. The Comments section has been added to the manuscript.

5. Manuscript and data are uploaded in Microsoft word file (.doc) and decomposable figures (.ppt), respectively.

6. The modified text is highlighted in red font.

7 The conflict of interest statement has been signed and uploaded.

8 The supplementary tables and figures are referenced in the article text.

Table S1 (see page 8 line 35)
Table S2 (see page 10 line 23)
Table S3 (see page 11 line 9)

Figure S1 (see page 10 line 14)
Figure S2 (see page 11 line 8)

The manuscript has been revised and improved according to the suggestions of the reviewers:

Reviewer 1 (02446101)

We greatly appreciate the kind and highly positive comments from this reviewer. The revised manuscript has been significantly improved for language and readability.

Reviewer 2 (00742043)

We are pleased that the reviewer found the results of our study interesting. We also thankful for the suggestions, which were helpful in improving the manuscript.

1. **The statistical analysis and P value should be shown in the Fig.4C, Fig 5B and Fig 7B:** We have revised the Figures 5 and 7 by adding p-values as suggested by the reviewer. However, quantitation of a single western blot precludes calculation of p-values for the Figure 4.

2. In Fig.6; The 3-D grown ESCs formed teratomas in mice should be shown by immunohistochemistry with the stainings of 3 germ layer markers: We have performed additional experiments to differentiate 3-D scaffold grown ESCs *in vitro* for immunofluorescence staining assays, which showed presence of cells of all three germ layers (Figure 7A).

3. Fig. 7; The differentiation of 3-D grown ESCs into selected lineages such as muscle cell and neuron should be shown in specific markers at protein level: Since our qRT-PCR results showed expression of cell-specific markers via directed differentiation of ESC-derivatives, we have now performed immunofluorescence staining of these cells to detect positive expression of all three germ layers at the protein level (Figure 7A). This further confirms that the pluripotency and differentiation potential of ESCs was maintained during long-term 3-D culture in the scaffold.

Reviewer 3 (00504335)

We are very grateful for the reviewer comments regarding the novelty of our findings.

1. The authors should clearly demonstrate the increase in the number of living cells per ml and compare the number of ESC after one or two weeks in their 3D system with classical 2D system:
We have performed additional experiments to show the proliferation rate of 3-D scaffold grown ESCs and compared them to 2-D cultured ESCs (Figure S1). These results further demonstrate the advantages of ESCs in this 3-D culture system. Although, 3-D cultured cells had a slower growth rate in comparison to 2-D cultured cells (approximately 36 hours compared to 12 hours, respectively), 3-D cells did not require passaging to avoid differentiation even after extend growth periods.

2. Exhaustion of medium: As stated in the Materials and Methods (page 7, line1-2), the medium was

changed every 3 to 4 days or as needed.

3. What was concentration of cells per ml after 3 weeks, if the original cell concentration was 1 or even 4 x 10(6)/ml? The doubling time of ESCs in the scaffold was observed to be about 36 hours (Figure S1). ESCs at a concentration of $2x10^6$ cells would grow to approximately $8x10^9$ cells in 3 weeks (including the lag period) using the formula for exponential cell growth $N = N_o \times 2^g$, where N is the final cell number, N_0 is the initial cell number ($2x10^6$), and g is the number generations.

I am pleased that the revised manuscript is much improved. I hope you will find our response to the reviewer comments and suggestions appropriate and the manuscript acceptable for publication.

Best regards,

G. Rasul Chaudhry