Stanford University

SUNDARI CHETTY
Assistant Professor
Department of Psychiatry and Behavioral Sciences
chettys@stanford.edu

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Dear Drs. Anna Justis and Phillip Steindel,

Thank you very much for your and the reviewers' helpful feedback on our manuscript. Please find attached a significantly revised manuscript (JoVE59833) entitled "**Transient treatment of human pluripotent stem cells with DMSO to promote differentiation**". We have conducted more experiments and added new data that directly address your and the reviewers' comments.

Please also find attached a response letter that addresses your comments as well as the concerns raised by each of the reviewers.

The main additions in the revised manuscript are as follows:

1. We have added new data quantifying the percentage of differentiated cells in the control and DMSO-treated conditions for all figures (Figures 2-5). This data confirms our initial findings that the DMSO treatment significantly enhances the differentiation capacity of human pluripotent stem cells (hPSCs) across multiple lineages.

2. We have conducted new experiments and added new data quantifying cell viability in control and DMSO-treated hPSCs (Figure 1). This data shows no significant difference in cell viability in control and DMSO-treated hPSCs, indicating that a 24h 1-2% DMSO treatment is not toxic to hPSCs. Control and DMSO-treated hPSCs also reach the same degree of confluence within 24h of removing the DMSO treatment. We have included this additional clarification in the manuscript and also emphasize that the DMSO treatment activates checkpoint controls and promotes cell cycle arrest in G1 (Chetty et al., 2013) to slow down cell proliferation and promote differentiation.

3. We have also provided additional clarification of the protocols and steps represented in the manuscript. All changes are tracked throughout the manuscript.

4. Finally, we have ensured the text, figures, and supporting materials are in accordance with the JoVE formatting guidelines following your recommendations.

In summary, these results confirm that a transient DMSO treatment has a significant impact on the differentiation potential of pluripotent stem cells. We have now conducted a comprehensive assessment of hPSC differentiation following a 24h DMSO treatment. The evidence firmly establishes that a simple DMSO treatment allows differentiation of hPSCs towards all germ layers and more mature terminal cell types, significantly relaxing current constraints in the stem cell field.

Thank you again for your valuable feedback, which improved this work significantly. We look forward to hearing from you about the revised manuscript.

Sincerely,

Sundari Chetty

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Department of Psychiatry and Behavioral Sciences, Stanford University School of Medicine
T 510.520.2328