Response to Comments by Academic Editor

Thank you very much for your interest in our manuscript and helpful comments and suggestions. Please find attached a revised manuscript. The following is a point-by-point response to the concerns you raised; editor's comments shown in bold, followed by our responses.

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have reviewed the manuscript and ensured there are no spelling and grammar errors.

2. Please include email addresses for all authors in the manuscript.

The email addresses for all authors are now provided in the manuscript.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Matrigel, Parafilm

We have revised the manuscript and ensure there is no use of commercial language.

Protocol:

1. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We have highlighted 2.75 pages or less of the Protocol to be represented in the video.

2. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

We ensure that we have described how each step/substep is performed and have also included references to published material for any protocols described.

Figures and Tables:

1. Figures 1, 4B: Please include scale bars here.
2. Figures 2,3: Please describe the scale bars in the corresponding legends.

3. Figure 2: Please use 'h' as an abbreviation instead of 'hrs'.

These modifications have been made to the corresponding figures.

Discussion:

1. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3–6 paragraphs with citations:
a) Critical steps within the protocol
b) Any limitations of the technique

We have revised the Discussion to describe any critical steps within the protocol and discuss any limitations of the technique.

References:

1. Please do not abbreviate journal titles.

We ensure journal titles are not abbreviated in the references.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We ensure that the Table of Materials includes information on all materials, reagents, and equipment mentioned in the Protocol.

2. Please include lot numbers for antibodies.

We have not observed any lot to lot variation in the antibodies used in our study. We have included this note in the Table of Materials.

Response to Comments by Reviewer #1

Thank you for your very helpful comments and suggestions, which have greatly improved the paper. Please find attached a revised manuscript. The following is a point-by-point response to the concerns you raised; reviewer's comments shown in bold, followed by our responses.

Major Concerns:

1. Line 395 Did DMSO treatment affect cell proliferation/cell number? Differences in 1B between experimental and control groups should be quantitated.

We thank the reviewer for this suggestion. We have conducted additional experiments and show data that cell viability is not significantly different in the control and DMSO-treated conditions (results section for Figure 1). While a 2% DMSO treatment may reduce cell number 24h after the treatment, cells 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.

2. Line 412. Figure 2B. Differences between +/- DMSO treatment should be quantitated.

Thank you for this suggestion which helped strengthen and confirm our initial findings. Quantification for Figure 2B has now been provided.

3. DMSO effects in Figure 3B and 3D should be quantitated.

Quantification for Figure 3 has now been provided.

4. Figure 4B. Differences between DMSO-treated and non-treated in stages 4 and 5 should be quantitated.

Quantification for Figure 4B has now been provided.

5. Figure 5B,C,D lacks statistics.

The statistical information has been added for Figure 5B, C, and D.

Response to Comments by Reviewer #2

Thank you for your very helpful comments and suggestions, which have greatly improved the paper. Please find attached a revised manuscript. The following is a point-by-point response to the concerns you raised; reviewer's comments shown in bold, followed by our responses.

Major Concerns:

1) The authors proposed the doses between 1-2% to be screened for different hPSC lines. In the figure 1 is shown the morphology of hiPSC colonies in 2D and 3D. Fig 1A 2% shows a clear toxic effect. However, not any clear read-out is shown in this paper regarding the screening of the toxic effect of DMSO, that constitutes a weak point and should be reconsidered for this paper having the DMSO treatment in the center!

We thank the reviewer for this suggestion. We have conducted additional experiments and provide new data in the revised manuscript confirming that a low DMSO dose of 1-2% has minimal toxicity. To assess cell viability, we harvested cells treated with or without 1-2% DMSO and used the Countess II FL Automated Cell Counter to quantify viability. In brief, 10 µl of Trypan Blue was mixed with 10 µl of cells and loaded into the Countess II FL Automated Cell Counter. The percentage of viable cells was quantified by the automated system using the trypan blue exclusion assay. This data shows no significant difference in cell viability between control and DMSO-treated cells and is now provided in the revised manuscript (results section for Figure 1). We have also provided additional clarification to prior work demonstrating that the effects of DMSO treatment of hESCs and hiPSCs at a concentration of 1-2% results in minimal toxicity at these low doses (Chetty et al., 2013). DMSO-treated cells reach the same degree of confluence as control cultures once the DMSO treatment is removed. This additional clarification is now included in the revised manuscript.

2) The protocols for endoderm differentiation are exemplified in protocols 3.1 and 4.3. Both are using a first step with WNT or agonist (CHIR) and activin A treatment and a second step with only activin A treatment in order to generate the definitive endoderm. The figure 4A these 2 steps toward definitive endoderm are not well presented, without step 2. In fig 5 for the same protocol it is another variant (3 days of step 2). The "endocrine" protocol (table 2, Fig 4 and Fig 5) should be revisited, as it is confusing in many terms. The goal is to show the terminal differentiation toward the pancreatic endocrine beta-cells, via endoderm differentiation. It is an unnecessary repetition in fig 4 and 5 regarding the protocol.

We thank the reviewer for catching this typo and have corrected the schematic for the protocol in Figure 4A. We appreciate the reviewer's suggestion for improving clarity of the protocols illustrated in the manuscript--we have replaced Figure 5A with a simplified schematic of the protocol to avoid unnecessary repetition. We have also revised the methods for 4.3 for clarity.

3) While in fig 2 SOX17 staining shows a difference after the DMSO treatment, in Fig 4 FOXA2 and PDX2 stainings show no differences in definitive endoderm and pancreatic progenitor specification, proved (only) here also quantitatively (Fig5). The authors claim a difference in NKX6.1 and C-pep stainings (Fig 4), but no quantification is shown. In case this is real, how can authors explain the late effect of the DMSO-primed differentiation?

We thank the reviewer for this suggestion as it helped confirm and strengthen our initial findings. We have now provided quantification for Figure 2 and Figure 4. We apologize for not emphasizing that different cell lines were used in Figure 2 (HUES6) and Figure 4 and 5 (HUES8). HUES6 has been shown to be more refractory towards differentiation whereas HUES8 differentiates into endodermal cells more readily (Osafune et al., 2008; Bock et al, 2011). This distinction is now clarified in the manuscript and figure legends. These differences likely account for the later effect of DMSO in HUES8 cells, which already have high propensity towards endodermal lineage and therefore do not initially benefit as greatly from DMSO pretreatment. Based on our prior work (Chetty et al., 2013), we have provided additional mechanistic data in the discussion of the manuscript explaining that the long term improvements in differentiation may be due to activation of Rb and effects on the cell cycle elicited by the DMSO treatment.

Minor Concerns:

1) An unclear aspect is the nomenclature of the embryoid bodies in Fig 1B. Usually this name is attributed to spheroids containing the cells of the 3 germ layers spontaneously differentiated from PSC in suspension culture. After 1 day treatment with DMSO the name hPSC in 3D culture is more realistic. Fig 1B does not show an "uniform embryoid body formation" in any treatment. Also the expression "EB colony formation" at line 401 is "novel".

Thank you for correcting the nomenclature used in the manuscript. The use of “embryoid bodies” has been corrected throughout the manuscript and figures to refer to “3D cell spheres”. The use of “uniform embryoid body formation” has also been removed from the manuscript.

2) Protocol 3.3 (line 235) is designed for neural differentiation, generating neuroectodermal cells, as the first steps in the protocols 4.1 and 4.2. SOX1 is a late marker in human neural precursors, after PAX6, that is shown also in figs 2 (ectoderm) and 3 (NPCs). The patterning differences in these protocols are not clearly pointed.

While previous studies had suggested that PAX6 preceded SOX1 expression, early induction of SOX1 prior to PAX6 was observed in the protocol referenced for the ectoderm differentiation (Chambers et al., 2009) in Figure 2 of our study. In fact, Chambers et al., 2009 note that the earliest neural marker expressed in their culture system was Sox1 preceding induction of Pax6. Sox1 expression peaks 3 days after differentiation in Chambers et al., 2009, which corresponds with the presence of SOX1 positive cells in Figure 2 of our study. The dual-SMAD inhibition neuroectodermal protocols (Chambers et al., 2009; Tchieu et al., 2017) illustrated in our manuscript are widely used and have been shown to reliably generate a broad repertoire of hPSC–derived neural cell types with further directed differentiation.

3) The protocols for mesoderm (3.2) stops after 1 day treatment with activin A and Wnt to mesendoderm. Some clarifications regarding the comparison with protocol 3.1 as well as further fates would be necessary here (as in discussion line 447 regarding the beating cardiomyocytes).

The expression of the Brachyury gene is required for mesoderm formation and becomes activated as an immediate-early response gene by mesoderm-inducing factors, such as activin A (Smith et al., 1991). Thus, we used a simple and short 1-day treatment to illustrate the benefits of the DMSO treatment in promoting mesodermal induction. Also, as opposed to protocol 3.1, the protocol for mesoderm (3.2)

uses advanced RPMI medium which has less serum supplementation and is especially useful for therapeutic applications.

The protocol to generate cardiomyoctyes is an extended mesodermal protocol–we have included the references for these protocols (Zhang et al., 2008; Lian et al, 2012) in the revised manuscript.

4) The nomenclature of the human marker genes/proteins should be uniformly presented with capital letters: FOXA2, NKX6.1, PDX1, PAX6, OLIG1, NKX6.1

These edits have been made throughout the manuscript to be uniformly presented.

Thank you again for your and the reviewers' detailed comments. We feel that the revised article more clearly represents the significance and practical relevance of our findings, and we hope that it addresses the primary concerns raised.