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Transient treatment of human pluripotent stem cells with DMSO to promote differentiation

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March 18, 2019

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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TITLE:

Transient Treatment of Human Pluripotent Stem Cells with DMSO to Promote Differentiation

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KEYWORDS:

human pluripotent stem cells, differentiation, DMSO, retinoblastoma protein, cell cycle, cell fate

SUMMARY:

Generating differentiated cell types from human pluripotent stem cells (hPSCs) holds great therapeutic promise but remains challenging. PSCs often exhibit an inherent inability to differentiate even when stimulated with a proper set of signals. Described here is a simple tool to enhance multilineage differentiation across a variety of PSC lines.

ABSTRACT:

Despite the growing use of pluripotent stem cells (PSCs), challenges in efficiently differentiating embryonic and induced pluripotent stem cells (ESCs and iPSCs) across various lineages remain. Numerous differentiation protocols have been developed, yet variability across cell lines and low rates of differentiation impart challenges in successfully implementing these protocols. Described here is an easy and inexpensive means to enhance the differentiation capacity of PSCs. It has been previously shown that treatment of stem cells with a low concentration of dimethyl sulfoxide (DMSO) significantly increases the propensity of a variety of PSCs to differentiate to different cell types following directed differentiation. This technique has now been shown to be effective across different species (e.g., mouse, primate, and human) into multiple lineages, ranging from neurons and cortical spheroids to smooth muscle cells and hepatocytes. The DMSO pretreatment improves PSC differentiation by regulating the cell cycle and priming stem cells to be more responsive to differentiation signals. Provided here is the detailed methodology for using this simple tool as a reproducible and widely applicable means to more efficiently differentiate PSCs to any lineage of choice.

INTRODUCTION:

The use of pluripotent stem cells has led to numerous advancements in biomedical research, including the fields of regenerative medicine and stem-cell based therapies, disease modeling, and drug screening. It has also led to the overall prospect of more translatable research and personalized medicine. The advent of induced pluripotent stem cell (iPSC) technology over 20 years ago has allowed researchers to develop pluripotent stem cells from somatic tissues and differentiate them into functional cell types to study a variety of pathologies, including cardiovascular, neurological, and immunological diseases. Although significant strides have been made in stem cell differentiation technology, challenges in effectively differentiating human embryonic stem cells (hESCs) and iPSCs still persist, limiting the widespread use of stem cell technology across different research programs. Inherent variability across different cell lines and clones continues to pose obstacles for differentiating stem cell lines to desired linages[1]. Furthermore, deriving mature, terminally differentiated functional cells from hPSCs remains a tedious and inefficient process across many lineages. In fact, cells differentiated from hPSCs often fail to terminally differentiate into functional cells[2]. In further moving stem cell-based therapies to use in patients, there is a need to improve and ensure the efficacy of cells that are generated from hPSCs.

Our lab has established a quick, inexpensive tool to significantly enhance the efficiency of differentiating both iPSCs and ESCs into mature cell types. We found that pretreatment of hiPSCs and hESCs with the commonly used reagent dimethyl sulfoxide (DMSO) for 24 h to 48 h prior to directed differentiation results in a marked improvement in stem cell differentiation capacity. Treatment with DMSO increases the proportion of hiPSCs and hESCs in the early G1 phase of the cell cycle and activates the retinoblastoma protein (Rb)[3], a critical regulator of cell proliferation, survival, and differentiation[4]. In more recent work, it has been found that Rb and its family members are required for the pro-differentiation effects of DMSO, such that transient inactivation of Rb suppresses the effects of DMSO, while constitutive activation of Rb in a transient manner enhances DMSO's effects[5]. Analogous to the cell cycle during embryonic development, the cell cycle of ESCs and iPSCs is characterized by an abbreviated G1 phase that promotes self-renewal[6-8]. This abbreviated G1 phase allows for more unrestricted proliferation but limits the potential for differentiation[4,9]. By promoting growth arrest in G1 and activating checkpoint controls in the cell cycle of hESCs and iPSCs, the DMSO treatment primes cells for cell fate changes following directed differentiation.

To date, DMSO pretreatment has been shown to improve the differentiation capacity to all three germ layers in over 30 control and disease-specific human ESC and iPSC cell lines[3,5] as well as the differentiation of stem cells and other cell lines to a variety of other mature cell types in subsequent studies[10-28] (**Table 1**). Furthermore, DMSO treatment has been shown to be effective in enhancing differentiation of non-human primary cells[21,23] (e.g., mouse, primate, rabbit), suggesting shared mechanisms across species. Lastly, DMSO pretreatment has also been extended to gene editing technology, with one particular study showing that 24 h DMSO pretreatment of hESCs/iPSCs significantly increased the ability of Clustered Regularly

Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein-9 (Cas9)-mediated editing efficiency of non-coding DNA without incorporating unintended mutations[29]. Provided here is a detailed methodology of the DMSO pretreatment of hESCs and iPSCs for applications in stem cell biology and directed differentiation.

PROTOCOL:

1. Stem cell maintenance

NOTE: The cell maintenance protocol described below applies to pluripotent stem cells (PSCs) maintained in an adherent monolayer. Media, other reagents, and cell culture plates used prior to DMSO treatment can be adjusted as needed. For all the following protocols in this manuscript, cells should be handled under a biological safety cabinet.

1.1. Coat sterile, 6 well, tissue culture-treated plates with a pluripotent stem cell-qualified matrix or substrate prepared per the manufacturer's instructions and incubate for at least 1 h in a CO_2 incubator (5% CO_2, humid atmosphere). Coated plates can be film wrapped and stored at 4 °C for up to one week.

1.2. Thaw the cryopreserved PSCs in a 37 °C water bath. Sterilize vial with ethanol prior to introduction to the biological safety cabinet, then immediately transfer the cells by pipetting to a sterile conical tube containing 5–10 volumes of prewarmed stem cell media.

1.3. Centrifuge the cells at 300 x *g* for 5 min at room temperature (RT).

1.4. Aspirate the media and gently resuspend the cell pellet in 1 mL of stem cell media supplemented with a 10 µM ROCK inhibitor, such as Y-27632.

1.5. Aspirate the culture matrix from the plate and seed the cells at the desired density, typically 0.5–1 x 10^6 cells per well in at least 2 mL of stem cell media per well.

NOTE: The plating density can vary across different cell lines, and clones and should be optimized accordingly.

1.6. Maintain the cells by replacing with prewarmed stem cell media daily. Split the cells at roughly 70%–80% confluency or when the cell colonies begin to make contact.

NOTE: The ideal confluency for splitting cells should be optimized per cell line, as some cell lines vary among growth rate and sensitivity to over- or under-confluence.

1.7. For splitting cells, aspirate the media and wash the cells once with sterile PBS. Incubate the cells with 1 mL of a dissociation enzyme solution per well for 5–10 minutes at 37 °C.

1.8. Wash and resuspend the cells with prewarmed stem cell media and transfer to a sterile conical tube with 5–10 volumes of stem cell media. Follow steps 1.3–1.7 to plate the cells.

2. DMSO pretreatment

NOTE: When plating the cells for DMSO pretreatment prior to differentiation, the starting plating cell density should be optimized with consideration of the typical growth rate of the stem cell line as well as the differentiation protocol being used. Validate pluripotency using conventional markers, as necessary. Cells should be passaged at least 1x–2x after initial thawing prior to differentiation.

2.1. 2D culture differentiation

2.1.1. When the cells reach an appropriate confluency, prepare coated plates, dissociate the cells, and prepare a single-cell suspension as described above.

2.1.2. Count the live cells using a hemocytometer or automatic cell counter including trypan blue or another viability marker.

2.1.3. Plate the cells onto a coated 6 well plate at 0.5–1 x 10^6 cells per well in stem cell media with the 10 µM ROCK inhibitor.

NOTE: For the cell lines tested in our laboratory, these densities typically resulted in 80%–90% confluent cells within the 24 h DMSO pretreatment.

2.1.4. Allow cells to incubate for 24 h at 37 °C in a CO_2 incubator (5% CO_2, humid atmosphere).

2.1.5. Prepare 1%–2% DMSO in prewarmed stem cell media (e.g., 100 µL of DMSO in 10 mL of media = 1% DMSO solution, or 200 µL DMSO in 10 mL of media = 2% DMSO solution).

2.1.6. After 24 h incubation, aspirate the media from cells and replace it with DMSO solution.

2.1.7. Allow the cells to incubate for 24 h to 48 h at 37 °C in a CO_2 incubator (5% CO_2, humid atmosphere) prior to differentiation.

NOTE: Typically, a 24 h DMSO treatment is sufficient across a majority of human ESC and iPSC lines. Cell lines with very slow growth rates (long doubling times) can benefit from the 48 h incubation with DMSO. For a 48 h incubation with DMSO, media can be replaced with fresh stem cell media with 1%–2% DMSO after the first 24 h of treatment.

2.2. 3D culture differentiation:

2.2.1. When the cells reach an appropriate confluency, dissociate and collect the cells in a cell suspension as described above.

2.2.2. Count the live cells using a hemocytometer or automatic cell counter including a viability marker.

2.2.3. Plate the cells in an uncoated, low-attachment 6 well plate at 0.5–1 x 10^6 cells per well in stem cell media with 10 µM ROCK inhibitor.

NOTE: For the cell lines tested in our laboratory, these densities typically resulted in 3D hPSC sphere formation within 24 h of setting cells.

2.2.4. Allow cells to incubate for 24 h at 37 °C in a CO_2 incubator (5% CO_2, humid atmosphere).

2.2.5. Prepare 1%–2% DMSO in prewarmed stem cell media (e.g., 100 µL of DMSO in 10 mL of media = 1% DMSO solution, or 200 µL of DMSO in 10 mL of media = 2% DMSO solution).

2.2.6. Replace the media following standard procedures (e.g., tilt the plate at a 30°–45° angle to allow cell spheres to settle at the bottom of the well; transfer cells to a sterile conical tube and allow cell spheres to settle at the bottom of the tube; or gently collect cells in suspension using a 5 or 10 mL pipette into a sterile conical tube and centrifuge cells at 300 x *g* for 5 min at RT).

2.2.7. Aspirate the media from cells and replace it with DMSO solution, pipetting gently.

2.2.8. Allow cells to incubate for 24 h to 48 h at 37 °C in a CO_2 incubator (5% CO_2, humid atmosphere) prior to differentiation.

NOTE: Typically, a 24 h DMSO treatment is sufficient across a majority of human ESC and iPSC lines. Cell lines with very slow growth rates (long doubling times) can benefit from the 48 h incubation with DMSO. For a 48 h incubation with DMSO, media can be replaced with fresh stem cell media with 1%–2% DMSO after the first 24 h of treatment.

3. Differentiation to primary germ layers

NOTE: The following describes methods previously shown to be effective in our laboratory for PSCs grown in a monolayer on 6 well plates. Any differentiation protocol of choice should be used after the DMSO treatment to promote differentiation into desired lineages. Remove DMSO solution after a 24–48 h treatment and proceed with differentiation following standard protocols.

3.1. **Endoderm differentiation** (adapted from Kroon et al.[30])

3.1.1. Pretreat cells with DMSO as described above for 2D cultures.

3.1.2. Prepare Wnt3a and Activin A stock solutions.

3.1.3. Prepare Day 1 endodermal differentiation media by adding Wnt3a to a final concentration of 20 ng/mL and Activin A to a final concentration of 100 ng/mL to the appropriate volume of prewarmed RPMI media.

3.1.4. After DMSO pretreatment, aspirate media from the cells and replace it with Day 1 media (e.g., 2 mL per well of a 6 well plate).

3.1.5. Allow cells to incubate for 24 h at 37 °C in a CO_2 incubator (5% CO_2, humid atmosphere).

3.1.6. Prepare Days 2 and 3 endodermal differentiation media by adding Activin A to a final concentration of 100 ng/mL to the appropriate volume of prewarmed RPMI media.

3.1.7. Aspirate media from the cells and replace it with Day 2 media (e.g., 2 mL per well of a 6 well plate).

3.1.8. Allow the cells to incubate for 24 h at 37 °C in a CO_2 incubator (5% CO_2, humid atmosphere).

3.1.9. Aspirate media from the cells and replace with Day 3 media (e.g., 2 mL per well of a 6 well plate).

3.2. **Mesoderm differentiation** (adapted from Zhang et al.[31])

3.2.1. Pretreat the cells with DMSO as described above for 2D cultures.

3.2.2. Prepare Wnt3a and Activin A stock solutions.

3.2.3. Prepare mesodermal differentiation media by adding Wnt3a to a final concentration of 20 ng/mL and Activin A to a final concentration of 100 ng/mL to the appropriate volume of prewarmed advanced RPMI media.

3.2.4. After DMSO pretreatment, aspirate media from cells and replace with differentiation media (e.g., 2 mL per well of a 6 well plate).

3.2.5. Allow cells to incubate for 24 h at 37 °C in a CO_2 incubator (5% CO_2, humid atmosphere).

3.3. **Ectoderm differentiation** (adapted from Chambers et al.[32])

3.3.1. Pretreat the cells with DMSO as described above for 2D cultures.

3.3.2. Prepare Noggin and SB431542 stock solutions.

3.3.3. Prepare ectodermal differentiation base media by dissolving knockout serum replacement (KOSR) to a final concentration of 10% in knockout DMEM.

NOTE: Prepare enough base media for 3–4 days of media change.

3.3.4. Prepare ectodermal differentiation media by adding Noggin to a final concentration of 500 ng/mL and SB431542 to a final concentration of 10 µM to the appropriate volume of prewarmed KOSR/knockout DMEM.

3.3.5. After DMSO pretreatment, aspirate media from cells and replace with differentiation media (e.g., 2 mL per well of a 6 well plate).

3.3.6. Allow cells to incubate for 3–4 days at 37 °C in a CO_2 incubator (5% CO_2, humid atmosphere), replacing media daily with freshly added differentiation factors.

4. Differentiation to progenitor cell types

NOTE: The following describes methods previously shown to be effective in our laboratory for PSCs grown in a 2D or 3D cultures. Any differentiation protocol of choice should be used after the DMSO treatment to promote differentiation into desired lineages. Remove DMSO solution after a 24–48 h treatment and proceed with differentiation following standard protocols.

4.1. **Neural progenitor cell differentiation** (adapted from Tchieu et al.[33])

4.1.1. Prepare 6 well plates by coating with a pluripotent stem cell-qualified reduced growth factor matrix or substrate, per the manufacturer's instructions, for at least 1 h in a CO_2 incubator (5% CO_2, humid atmosphere). Coated plates can be film wrapped and stored at 4 °C for up to 1 week.

4.1.2. Plate PSCs as described above at density of 0.5–1 x 10^6 cells per well in stem cell media containing a ROCK inhibitor.

4.1.3. Pretreat cells with DMSO as described above for 2D cultures.

4.1.4. Prepare small chemical inhibitors LDN193189, SB431542, and XAV939 stock solutions.

4.1.5. Prepare Days 1–3 neuroectoderm differentiation media by supplementing Essential 6 Media with 500 nM LDN193189, 10 µM SB431542, and 2 µM XAV939.

4.1.6. After DMSO pretreatment, aspirate the media and replace with Days 1–3 neuroectoderm media (e.g., 2 mL per well of a 6 well plate). Change media daily.

4.1.7. Prepare Days 4–12 neuroectoderm differentiation media by supplementing Essential 6 Media with 500 nM LDN193189 and 10 µM SB431542.

4.1.8. On day 4 of differentiation, aspirate the media and replace with Days 4–12 neurodectoderm media. Change the media daily.

4.1.9. After 12 days of differentiation, differentiated cells should express appropriate markers of neural progenitor cells (NPCs). NPCs can be further maintained in neural media containing DMEM/F-12, 2% B-27, 1% N-2, and supplemented with 10 µg/mL basic fibroblast growth factor (bFGF). Passage the NPCs when confluent using a cell detachment solution, plating NPCs at 0.5–1 x 10^6 cells per well.

4.2. **Oligodendrocyte progenitor cell differentiation** (adapted from Douvaras and Fossati[34])

4.2.1. Plate the PSCs as described above at a density of 1 x 10^5 per well on coated 6 well plates in stem cell media containing a ROCK inhibitor.

4.2.2. Pretreat cells with DMSO as described above for 2D cultures.

4.2.3. Prepare SB431542, LDN193189, all-trans retinoic acid (RA), and smoothened agonist (SAG) stock solutions.

4.2.4. Prepare Days 0–8 differentiation media by supplementing DMEM/F-12 with 10 µM SB431542, 250 nM LDN193189, and 100 nM RA.

4.2.5. After DMSO pretreatment, incubate cells with differentiation media for 8 days, changing media daily with freshly added differentiation factors (e.g., 2 mL per well of a 6 well plate).

4.2.6. On day 8, replace media with DMEM/F-12 containing 1X MEM non-essential amino acids (NEAA) solution, 1X L-glutamine, 2-mercaptoethanol, penicillin/streptomycin, and 1x N-2 supplemented 100 nM RA and 1 µM SAG. Change the media daily.

4.2.7. After 12 days of differentiation, differentiated cells should express appropriate markers of oligodendrocyte progenitor cells (OPCs).

4.3. **Endocrine progenitor cell differentiation** (adapted from Pagliuca et al.[35])

4.3.1. Seed PSCs at 6 x 10^5 cells/mL in stem cell media plus 10 µM ROCK inhibitor in 500 mL spinner flasks placed on a 9-position stir plate set at rotation rate of 70 rpm in a 37 °C incubator, 5% CO_2, and 100% humidity.

4.3.2. Allow clusters to settle at the bottom of the flask, aspirate the media, then pretreat with 1%–2% DMSO.

4.3.3. Prepare Activin A, Chir99021, KGF, Sant1, all-trans retinoic acid (RA), LDN193189, PdBU, XXI, Alk51, T3, and Betacelluin stock solutions.

4.3.4. Prepare differentiation base media based on formulation in **Table 3**.

4.3.5. After DMSO pretreatment, aspirate media and replace with S1 media supplemented with 100 ng/mL Activin A and 3 mM Chir99021 (e.g., 500 mL per flask). Allow incubation for 24 h.

4.3.6. On day 2, replace media with S1 media supplemented with 100 ng/mL Activin A. Allow incubation for 2 days.

4.3.7. On day 4, replace media with S2 media supplemented with 50 ng/mL KGF. Allow incubation for 3 days, changing media after the first 2 days (Day 6).

4.3.8. On day 7, replace media with S3 media supplemented with 50 ng/mL KGF, 0.25 mM Sant1, 2 mM RA, and 200 nM LDN193189. Allow incubation for 24 h.

4.3.9. On day 8, replace media with S3 media supplemented with 50 ng/mL KGF, 0.25 mM Sant1, 2 mM RA, 200 nM LDN193189, and 500 nM PdBU. Allow incubation for 24 h.

4.3.10. On day 9, replace media with S3 media supplemented with 50 ng/mL KGF, 0.25 mM Sant1, and 100 nM RA. Allow incubation for 5 days, changing media every 2 days (day 11 and 13).

4.3.11. On days 14 and 16, replace media with S5 media supplemented with 0.25 mM Sant1, 100 nM RA, 1 mM XXI, 10 mM Alk5i II, 1 mM T3, and 20 ng/mL betacellulin (4 days total incubation).

4.3.12. On days 18 and 20, replace media with S5 media supplemented with 25 nM RA, 1 mM XXI, 10 mM Alk5i II, 1 mM T3, and 20 ng/mL betacellulin.

5. Immunocytochemical validation of differentiation

NOTE: The following methods describes a general immunocytochemical protocol that can be adjusted as needed. Primary antibodies are those that have been previously validated in our laboratory. Other techniques for validation of differentiation can also be used (e.g., flow cytometry, qPCR, RNA sequencing, western blotting, functional assays, etc).

5.1. Immunolabeling cells

5.1.1. For 3D cultures in suspension, plate whole cell clusters or clusters dispersed into single-cell suspension onto coated plates for 18–24 h prior to fixation.

5.1.2. Aspirate media from adherent cells and wash briefly with PBS at RT on a shaker.

5.1.3. For cell fixation, aspirate PBS and incubate cells with 4% paraformaldehyde (PFA) in PBS for 20 min at RT on shaker.

CAUTION: PFA stock should be prepared under a fume hood due to its toxicity. Do not inhale and wear proper personal protective equipment.

5.1.4. Remove PFA and discard in the proper chemical waste container.

5.1.5. Wash cells 3x with PBS for at least 5 min per wash at RT on a shaker.

5.1.6. For cell permeabilization and blocking, incubate cells with 5% donkey serum prepared in 0.3% triton-x 100/PBS for 1 h at RT on a shaker.

5.1.7. Prepare primary antibody solution in the same solution used for permeabilization/blocking.

5.1.8. Incubate in primary antibody solution overnight at 4 °C on shaker.

5.1.9. After overnight incubation, wash cells 3x with PBS for at least 5 min per wash at RT on a shaker.

5.1.10. Prepare secondary antibody solution in permeabilization/blocking solution.

5.1.11. Allow to incubate in secondary antibody solution for 1 h at RT on a shaker.

5.1.12. Aspirate secondary antibody solution and wash cells 3x with PBS for at least 5 min per wash at RT on a shaker.

5.1.13. Incubate cells with DAPI or another preferred marker for appropriate incubation time, and rinse in PBS.

5.2. Image quantification

5.2.1. Acquire a minimum of three images per condition on a fluorescent microscope and/or with a high content screening platform.

5.2.2. Quantify the percent of positive cells for each marker by counting the total number of antibody stained cells and total cell numbers (based on DAPI/Hoechst nuclei staining) using unbiased imaging software (e.g., ImageJ) or an automated screening platform for analyses.

REPRESENTATIVE RESULTS:

Morphology of DMSO treated iPSCs

Human iPSCs derived from control subjects were cultured either in an adherent 2D monolayer or in 3D cell spheres in suspension. Approximately 24 h after initial plating, cells were treated with either 1% or 2% DMSO for 24 h in the maintenance medium. Representative brightfield

images after DMSO treatment are shown in **Figure 1**. Consistent with previous reports for iPSCs maintained in a monolayer[3], DMSO pretreatment resulted in a transient dose-dependent decrease in growth rate as compared to non-DMSO treated cells (**Figure 1A**). This decreased proliferation is associated with an increase in cell-to-cell contact, which is especially pronounced in the 2% DMSO treated cells displaying increased formation of more highly clustered cell colonies. In other cell types, DMSO-induced G1 arrest has been shown to be associated with increased expression of proteins involved in cell-cell interactions that support contact-inhibition induced growth arrest[36]. In iPSCs maintained as 3D cell spheres, the DMSO treatment similarly increased the number of cell spheres (**Figure 1B**). Furthermore, DMSO treatment also resulted in less variable 3D sphere sizes, which has been previously shown to be indicative of improved differentiation capacity of the cells[37]. Importantly, neither 1% or 2% DMSO resulted in cell toxicity, as measured by viability counts (n = 3; 2D culture % live = control: 80 ± 1.3; 1% DMSO: 82 ± 3.7, 2%: 81 ± 2.7; 3D culture % live = control: 81 ± 4.3; 1% DMSO: 82 ± 6.7, 2%: 82 ± 2.7). Overall, these results are consistent with the notion that DMSO treatment alters the cell cycle and growth patterns in cultured stem cells. These effects on growth inhibition are reversible when the DMSO is removed from the medium, as previously shown[3].

DMSO treatment improves the differentiation of ESCs to the primary germ layers

HUES6 hESCs were seeded on coated plates for 24 h followed by treatment with 2% DMSO for 24 h in the maintenance medium. Cells were then differentiated into the three primary germ layers following the treatment paradigms shown in **Figure 2A**[30-32]. Differentiated cells were then fixed and immunologically stained for prototypic markers of each respective germ layer (SOX17 for endoderm, brachyury for mesoderm, and SOX1 for ectoderm). As shown in **Figure 2B**, 24 h of pretreatment with 2% DMSO increased the proportion of cells expressing each respective germ layer marker. This is consistent with previous reports from our lab showing increased immunoreactivity, gene expression, as well as absolute number of differentiated cells towards all germ layers in stem cells treated with DMSO[3,5]. HUES6 is an hESC line with very low propensity for differentiation across all lineages[1], yet the DMSO treatment substantially improves its capacity to differentiate across all germ layers.

DMSO treatment improves the differentiation to progenitor cell types

To investigate the effect of DMSO on differentiation to CNS progenitor cell types, human iPSCs were differentiated to either neural progenitor cells (NPCs) or oligodendrocyte progenitor cells (OPCs). To generate NPCs, cells were pretreated with 2% DMSO for 24 h in the maintenance medium followed by 12 days of directed differentiation[33] (**Figure 3A**). As shown in **Figure 3B**, 2% DMSO pretreatment increased the expression of the NPC marker PAX6 as compared to control. Using another previously validated protocol[34] (**Figure 3C**), iPSCs were differentiated for 12 days into OPCs. Similar to NPCs, OPCs derived from iPSCs pretreated with 2% DMSO for 24 h demonstrated an increase proportion of cells expressing OPC markers OLIG2 (**Figure 3D**).

An initial DMSO treatment persists to enhance differentiation into mature cell types

To investigate the effect of DMSO on latter stages of a differentiation protocol, HUES8 hESCs were pretreated for 24 h with 2% DMSO prior to differentiation to β cells following a 20 day

directed differentiation protocol described in **Figure 4a**[35]. HUES8 were used as they have been previously shown to have a higher propensity towards endodermal lineage[1,38]. At the definite endoderm stage, the differentiated cells express SOX17 and FOXA2, definitive endoderm (DE) specific markers. With further differentiation into the pancreatic progenitors (PP_1) stage, differentiated cells express PDX1 and FOXA2, markers characteristic of pancreatic progenitor cells. At these stages of pancreatic cell differentiation, the efficiencies of induction into DE and subsequently into PP_1 were high for both control and DMSO-treated hESCs differentiated into each of these stages (**Figure 4B,** stages 1 and 3). Even though the HUES8 cell line has been noted to have increased propensity to differentiate into the endodermal lineage, as differentiation is induced further into the more specialized cell types at the terminal stages the DMSO-treated hESCs are much more likely to produce mature pancreatic endocrine cells. The efficiencies of generating PDX1/NKX6.1+ pancreatic progenitor cells, Neurogenin 3+ endocrine cells, and NKX6.1/C-peptide+ SC-β cells were substantially higher in the DMSO-treated hESCs (**Figure 4B,** stages 4 and 5). These results are in line with the NPC and OPC differentiation showing that DMSO enhances the differentiation potential to progenitor cell types and also demonstrates that the effect of DMSO is persistent in generating more specialized cell types. This is consistent with prior work, where we have shown that the initial 24 h DMSO treatment increases differentiation into terminal cell types across germ layers, including into neuronal cells as well as beating cardiomyocytes[31,39] in cell lines with high or poor propensities for differentiation[3].

Initial DMSO treatment improves hESC-derived cell function following *in vivo* transplantation

Previously, we have demonstrated the effectiveness of DMSO treatment in enhancing the differentiation of hESCs into functional pancreatic progenitor cells that later show a marked improvement in insulin secretion in vivo[3]. Using previously published protocols[3,30,40], HUES8 hESCs were treated with 1% DMSO for 24 h, differentiated into pancreatic progenitor cells, and transplanted into immunodeficient SCID-Beige mice to assess functionality (e.g., insulin secretion in response to a glucose challenge or KCl stimulation) (**Figure 5A**). While the efficiencies of differentiation into FOXA2+ (~90%) and PDX1+ (~75%) pancreatic progenitors were comparable between control and DMSO-treated hESCs (**Figure 5B**) for the HUES8 hESC line, the cells differentiated from hESCs following a 24 h 1% DMSO treatment had improved responsiveness to glucose and KCl stimulation following in vivo transplantation. Improvements in functionality were evident within 2 weeks post-transplantation (**Figure 5C**) and persisted up to at least 16 weeks post-transplantation (**Figure 5D**). Taken together, these results suggest that DMSO pretreatment not only increases the differentiation efficiency to germ layers, progenitor cells, and more mature cell types, but also that it persists to enhance functionality of the differentiated cells in vivo.

FIGURE LEGENDS:

Table 1: Summary of previously published work demonstrating the beneficial effects of DMSO treatment on differentiation.

Table 2: Components of endocrine progenitor cell differentiation base medias.

Figure 1: DMSO treatment alters the growth of hPSCs. (A) Representative brightfield images of hiPSCs plated in a monolayer after receiving no treatment (control) or treatment with 1% or 2% DMSO for 24 h. DMSO promotes a transient dose-dependent growth inhibition of iPSCs. **(B)** Representative brightfield images of hiPSCs plated on low-attachment plates to allow 3D sphere formation after receiving no treatment (control) or treatment with 1% or 2% DMSO for 24 h. DMSO treatment results in less variable 3D sphere formation compared to control. Scale bar = 500 μm.

Figure 2: DMSO treatment improves differentiation of hPSCs to primary germ layers. (A) Schematic of differentiation protocols used to generate the three primary germ layers. **(B)** Representative images of differentiated HUES6 hESCs immunolabeled for SOX17 (endoderm), brachyury (mesoderm), and SOX1 (ectoderm). Pretreatment with 2% DMSO for 24 h increased the differentiation efficiency across all three germ layers. Percentages of cells differentiating into SOX17+ endodermal, Brachyury (Brachy)+ mesodermal, or SOX1+ ectodermal cells following directed differentiation into each germ layer of control and DMSO-treated hESCs are noted with SEM of three biological replicates. Unpaired t-test: endoderm $p = 0.0003$; mesoderm $p = 0.047$; ectoderm $p = 0.015$. Scale bar = 50 μm.

Figure 3: DMSO treatment improves differentiation to neural progenitor cell types. (A) Schematic of differentiation protocol used to generate neural progenitor cells (NPCs). **(B)** Representative images of human iPSCs differentiated into NPCs immunolabeled for Pax6. 24 h of pretreatment with 2% DMSO increased the number PAX6 positive cells. Percentages of cells differentiating into Pax6+ NPCs following directed differentiation of control and DMSO-treated human iPSCs are noted with SEM of three biological replicates. Unpaired t-test: $p = 0.0225$. Scale bar = 200 μm. **(C)** Schematic of differentiation protocol used to generate oligodendrocyte progenitor cells (OPCs). **(D)** Representative images of human iPSCs differentiated into OPCs immunolabeled for OPC markers Olig2. 24 h of pretreatment with 2% DMSO increased the expression of both OPC markers compared to control. Percentages of cells differentiating into Olig2+ OPCs following directed differentiation of control and DMSO-treated human iPSCs are noted with SEM of four biological replicates. Unpaired t-test: $p = 0.0466$. Scale bar = 50 μm.

Figure 4: DMSO treatment enhances terminal differentiation potential of hPSCs. (**A**) Schematic of a ~20 day directed differentiation of HUES8 hESCs into terminally differentiated pancreatic endocrine cells. (**B**) Immunostaining for the indicated markers at each stage of differentiation following directed differentiation of untreated control cells and cells pretreated with 2% DMSO for 24 h. The initial DMSO treatment persists to increase differentiation into terminal endocrine cell types at the latter stages of directed differentiation. Percentages of cells differentiating into the indicated markers at each stage of differentiation following directed differentiation of control and DMSO-treated hESCs are noted with SEM of two to four biological replicates. Scale bar = 200 μm.

Figure 5: Initial DMSO treatment of hPSCs enhances glucose responsiveness following transplantation of pancreatic progenitor cells in vivo. (**A**) Schematic of directed differentiation

(~15 days) of HUES8 hESCs into pancreatic progenitor cells (PP_2) following no treatment (control) or a 24 h 1% DMSO treatment and subsequent transplantation (5 million cells) into immunodeficient SCID-Beige mice. (**B**) Percentage of cells differentiating into PDX1+ and FOXA2+ pancreatic progenitor cells following in vitro directed differentiation of control and DMSO-treated hESCs immediately before transplantation (n = 1). (**C**) Mean ELISA measurements of human insulin from the serum of mice following a low (2.5 mM) or high (15 mM) glucose challenge or potassium chloride (KCl) stimulation at (**C**) 2 weeks and (**D**) 16 weeks post-transplantation of pancreatic progenitor cells differentiated from control and DMSO-treated hESCs (error bars = SEM; n = 3 at 2 weeks and 16 weeks for control; n = 2 at 2 weeks and 16 weeks for DMSO). Two-way ANOVA: p = 0.0051 for control vs. DMSO at 2 weeks; p = 0.0116 for control vs. DMSO at 16 weeks. The mice studied at the different time points are different. Results are adapted from Chetty et al.[3].

DISCUSSION:

In summary, this protocol describes a simple and inexpensive tool to enhance the differentiation capacity of pluripotent stem cells (PSCs) to all primary germ layers, various types of specialized progenitor cells, and even functional, mature cell types in *in vitro* and *in vivo* settings. Illustrated are specific differentiation protocols that have been effectively reproduced in our laboratory as well as others, but any differentiation protocol of choice can be used following the DMSO treatment. As shown in **Table 1**, a number of laboratories have also demonstrated an enhancement of PSC differentiation after transient DMSO treatment using different paradigms to generate various other terminal cell types. Furthermore, although the methods here describe the use of human PSCs, the DMSO pretreatment can be utilized across species and has been shown to be effective in mouse, rabbit, and primate PSCs.

Although higher doses of DMSO are known to be cytotoxic, the low doses used in this method (1%–2%) for a transient period result in minimal cell death. While overall cell numbers immediately after DMSO treatment may decrease due to DMSO promotion of cell cycle arrest in the G1 phase of the cell cycle, previous studies show that cells are able to reach the same level of confluency as control cultures after removal of DMSO[3].

The percent and duration of DMSO pretreatment should be optimized per cell line. The treatment time should be adjusted with consideration for the cycling/doubling time of the cells. For example, mouse PSCs typically have much shorter cycling times of about 15 h; thus, DMSO treatment for 15 h for these cells is sufficient. Some labs have also found the DMSO treatment to be beneficial when continued during the differentiation protocol or at lower concentrations (see **Table 1**). It should be noted that some PSC lines are more amendable to differentiation to specific lineages. For example, HUES6 cells have been shown to be less permissive to differentiation and thus had marked improvement with DMSO treatment (**Figure 2**). Alternatively, HUES8 cells used in **Figure 4** and **Figure 5** have been shown to have a higher propensity towards endodermal differentiation; thus, fewer differences were shown between control and DMSO for differentiation at the initial stages towards definitive endoderm. Nonetheless, the enhancement of DMSO pretreatment is observed in later stages of

differentiation in this cell line (**Figure 4B**). The DMSO treatment is also versatile in that it is effective in both 2D and 3D cell cultures systems, it can be used with various types of coating material on cell culture plates, and it works in different types of maintenance medium that promote growth and expansion of hPSCs (e.g., mTeSR, E8, MEF conditioned media, etc).

More generally, these results suggest that the starting state of pluripotent stem cells has a strong influence on the propensity for initial differentiation as well as terminal differentiation into functional cell types. We have previously shown that the DMSO treatment functions through Rb in hPSCs[3,5]. Rb plays an important role in promoting terminal differentiation, cell survival, and the genetic stability of cells[41-44], and it may therefore explain the persistent effects on cells differentiated from DMSO-treated hPSCs. Targeting these early modes of regulation may place hPSCs on a better trajectory for differentiation and ultimately improve their utility for regenerative medicine.

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DISCLOSURES:

The authors having nothing to disclose.

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Figure 1

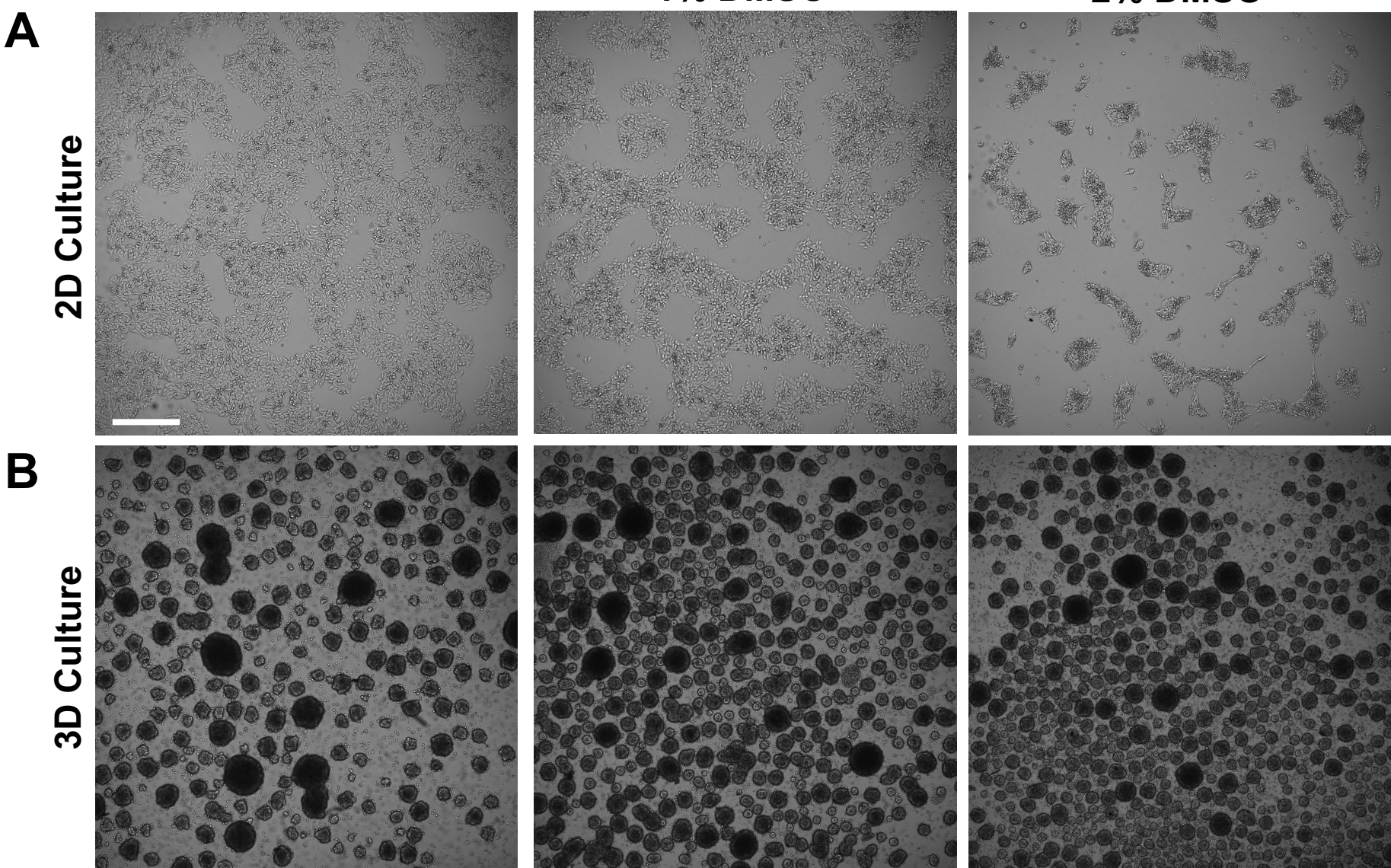

Figure 2

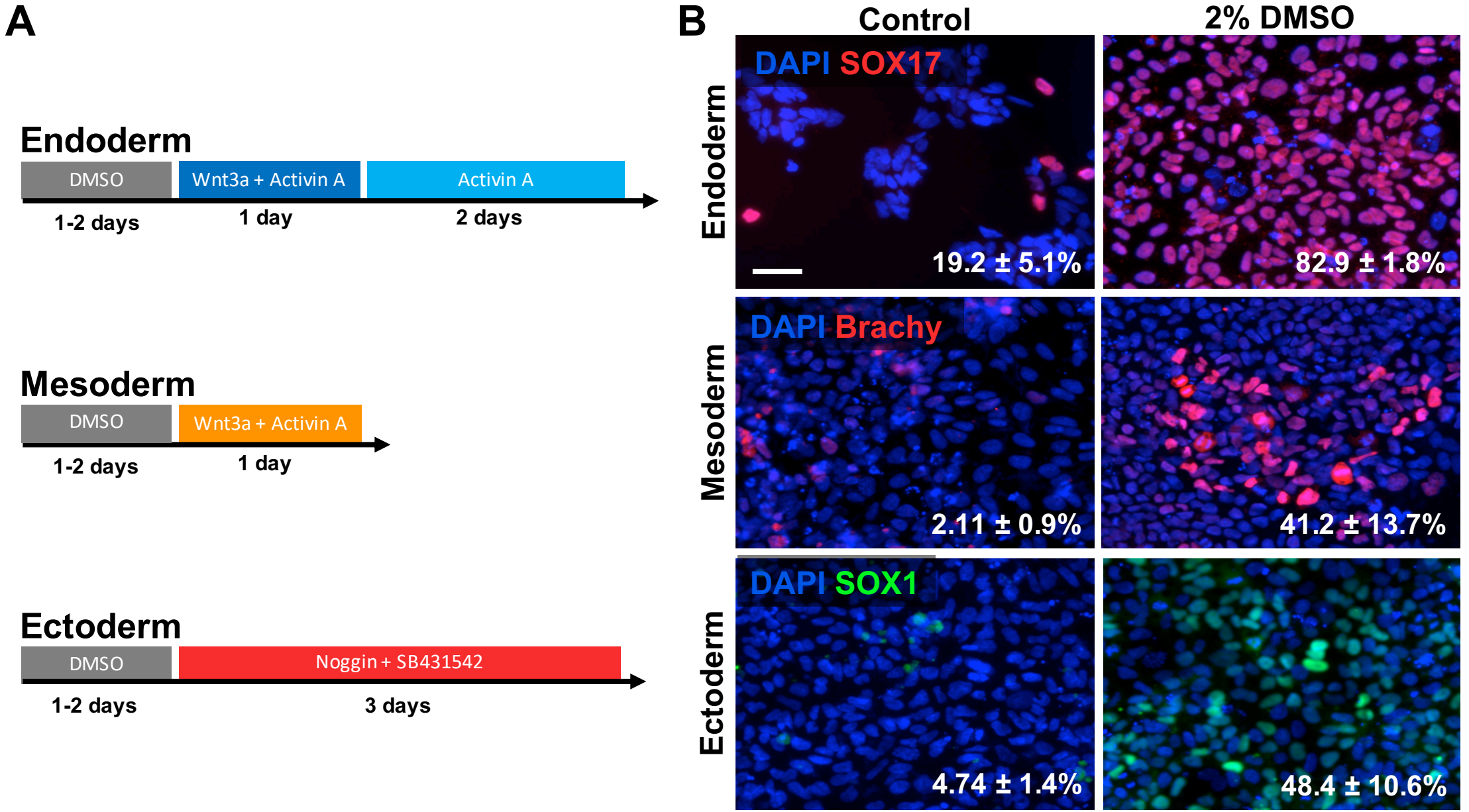

Figure 3

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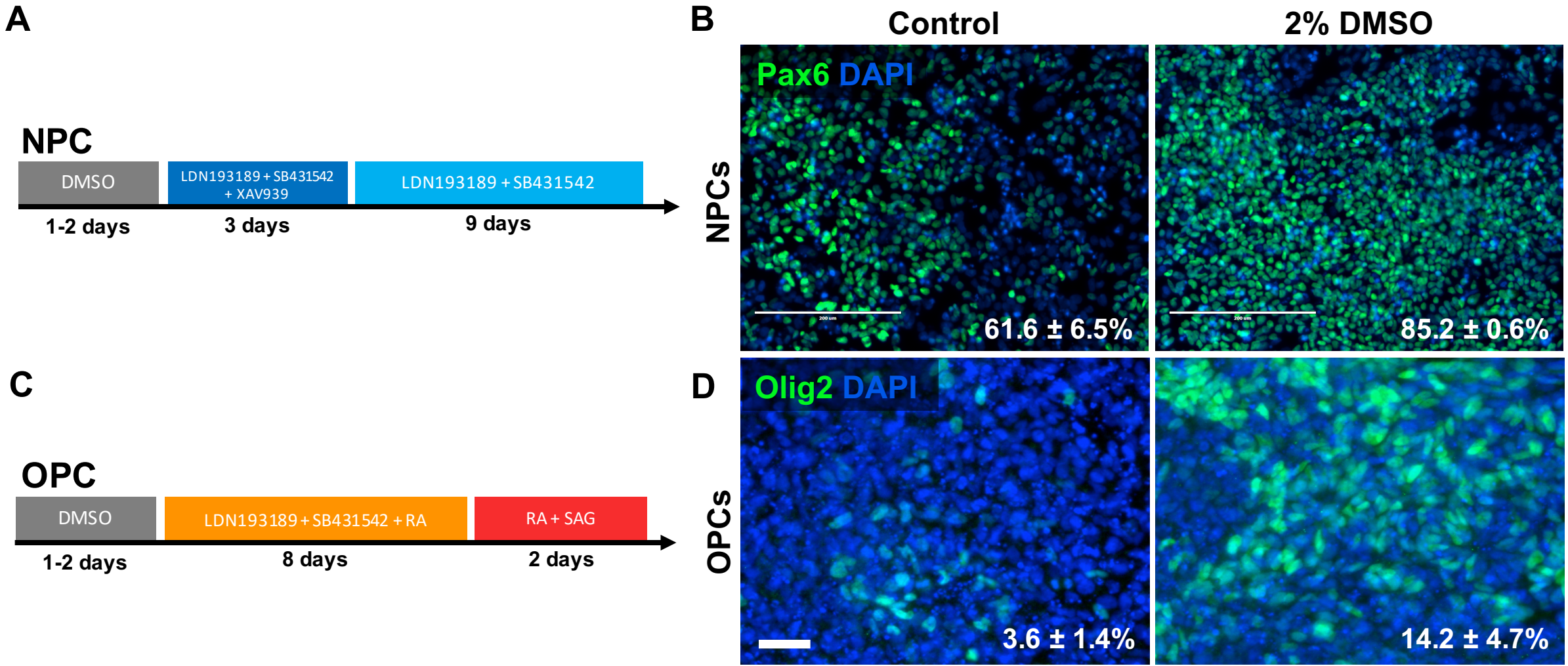
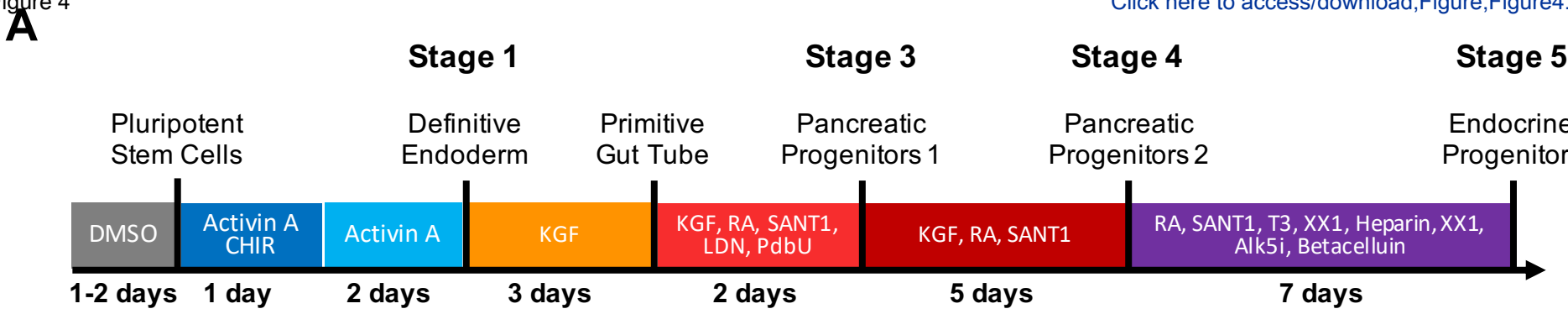


Figure 4



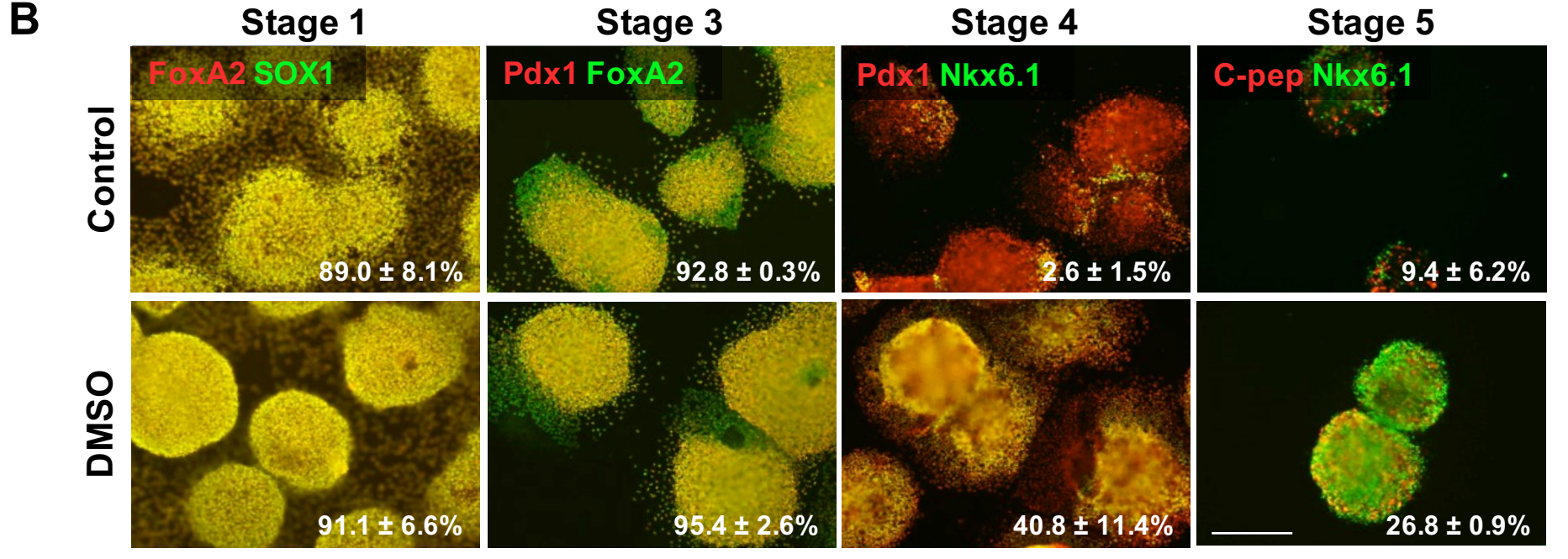


Figure 5

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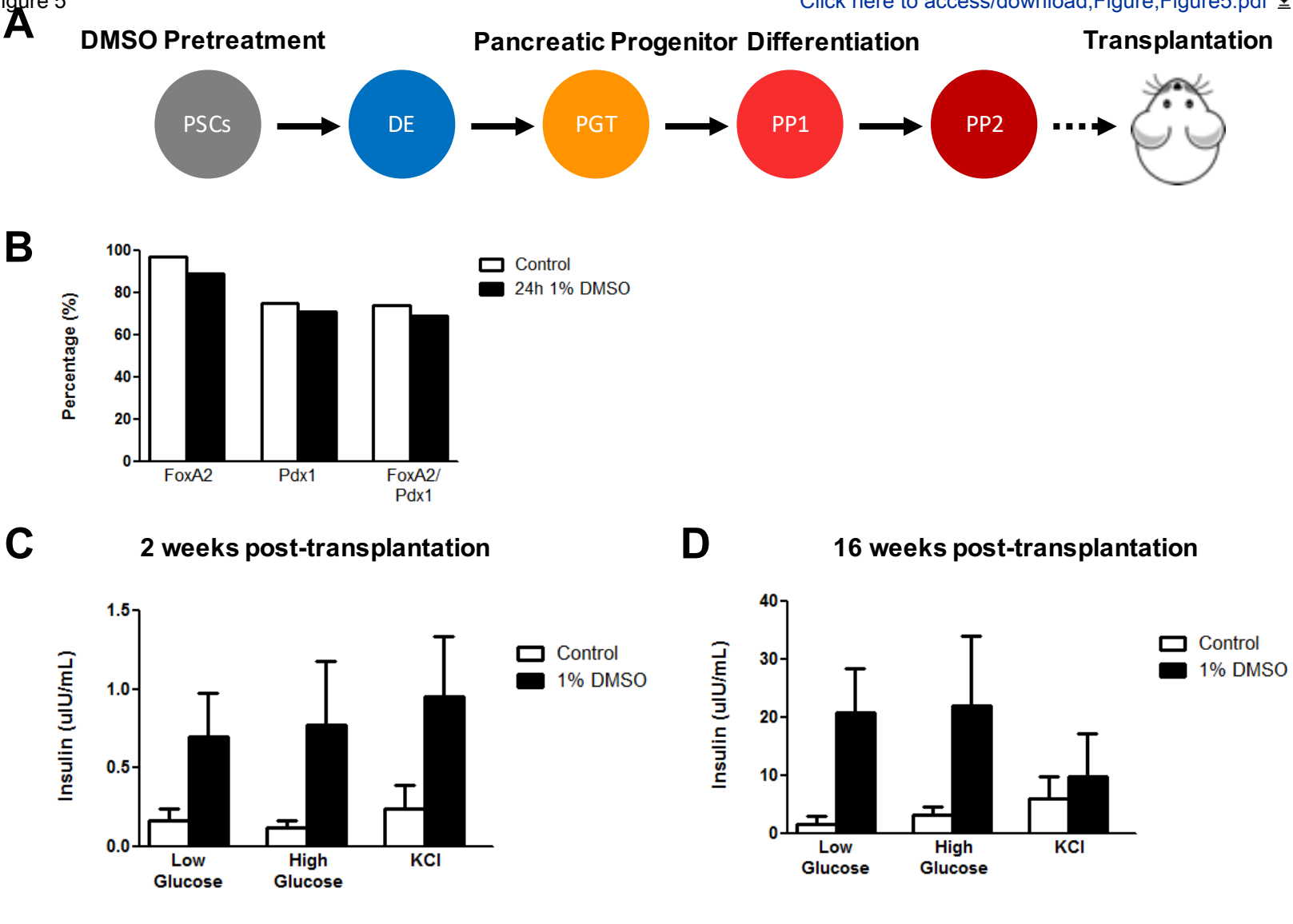

Table 1 Click here to access/download;Table;Table 1_v2.xlsx

Differentiated Cell Type	Starting Cell Type	%DMSO
Hepatic cells	ESC	1.0
	Hepatoma cell line	1.0
	ESC	1.0
	ESC	0.5
	Mesenchymal stem cells	0.1-2.0
	iPSCs	1.0
	ESC	1.0
	ESC	0.5
	Hepatoma cell line	1.0
	ESC	0.6
Primary germ layers	ESCs and iPSCs	0.1-2.0
	hESC	0.5
	hESC	0.1-2.0
Cardiac cells	ESCs and iPSCs	0.1-2.0
	P19 cells	1.0
	ESCs and iPSCs	1.0-2.0
	Fetal mesenchymal stem ce	0.8-1.0
Pancreatic cells	ESCs and iPSCs	0.1-2.0
	hESC	0.5
Smooth muscle cells	P19 cells	1.0
Endothelial cells	P19 cells	1.0
Enterocytes	iPSCs	0-1.6
Gut epithelium	iPSCs	0-1.6
Neural cells	Marmoset iPSC	0.05-2.0
Neutrophils	Leukemia cell line	1.25
Skeletal Myotubes	iPSCs	1.5
Cortical organoid	hiPSCs	1.0

Length of DMSO Treatment	Reference
8 days	Basma et al., 2008
Several days	Kanebratt and Andersson, 2008
7 days	Hay et al., 2009
10-14 days	Duan et al., 2010
7-21 days	Alizadeh et al., 2014
7 days	Kondo et al., 2014
4 days	Szkolnicka et al., 2014
5 days	Czysz et al., 2015
2-21 days	Nikolaou et al., 2016
Throughout	Vanhove et al., 2016
24 hours	Chetty et al., 2013
24 hours	Chetty et al., 2015
24 hours	Li et al., 2018
24 hours	Chetty et al., 2013
4 days	Choi et al., 2014
24-30 hours	van den Berg et al., 2016
24 hours	Deng et al., 2017
24 hours	Chetty et al., 2013
24 hours	Chetty et al., 2015
4 days	Choi et al., 2014
4 days	Choi et al., 2014
4 days	Ogaki et al., 2015
4 days	Ogaki et al., 2015
24 hours	Qiu et al., 2015
6-8 days	eimourian and Moghanloo, 2016
24 hours	Swartz et al., 2016
24 hours	Yoon et al., 2018

Table 2

	S1	S2	S3	S5
MCDB131 (L)	1	1	1	1
Glucose (g)	0.44	0.44	0.44	3.6
NaHCO3 (g)	2.46	1.23	1.23	1.754
FAF-BSA (g)	20	20	20	20
ITS-X (mL)	0.02	0.02	5	5
Glutamax (mL)	10	10	10	10
Vitamin C (mg)	44	44	44	44
Heparin (mg)	0	0	0	10
P/S (mL)	10	10	10	10

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2-mercaptoethanol	Gibco	21985023	
6-well Clear Flat Bottom TC-treated Multiwell Cell Culture Plate	Corning	353046	
9-Position stir plate	Chemglass	CLS-4100	
Accutase	Gibco	11105-01	
Activin A	R&D Systems	338-AC	
Advanced RPMI	Gibco	12633012	
Alk5i II	Axxora	ALX-270-445	
all-trans retinoic acid	Sigma-Aldrich	R2625	
anti-Brachyury	R&D Systems	AF2085	No variablity observed across different lot numbers
anti-C-peptide	Developmental Studies	GN-ID4	No variablity observed across different lot numbers
anti-FoxA2	Millipore	07-633	No variablity observed across different lot numbers
anti-Nkx2.2	University of Iowa, Dev	74.5A5	No variablity observed across different lot numbers
anti-Nkx6.1	University of Iowa, Dev	F55A12-supernatant	No variablity observed across different lot numbers
anti-Olig2	EMD Millipore	MABN50	No variablity observed across different lot numbers
anti-Pax-6	Biolegend	901301	No variablity observed across different lot numbers
anti-Pdx1	R&D Systems	AF2419	No variablity observed across different lot numbers
anti-SOX1	R&D Systems	AF3369	No variablity observed across different lot numbers
anti-SOX17	R&D Systems	AF1924	No variablity observed across different lot numbers
B-27 Supplement, minus Vitamin A	Gibco	12587010	
basic fibroblast growth factor	Gibco	PHG0264	
Betacellulin	Thermo Fisher Scientific	50932345	
Chir99021	Stemgent	04-000-10	
CMRL 1066	Corning	99-603-CV	
Counter	Scientific	AMQAF1000	
D-(+)-Glucose	Sigma	G7528	
DAPI	Invitrogen	D1306	
Disposable Spinner Flasks	Corning, VWR	89089-814	
DMEM/F-12	Gibco	11320033	
DMSO	Sigma-Aldrich	D2650	
Essential 6 Media	Gibco	A1516501	
FAF-BSA	Proliant	68700	

FGF7	PeproTech	100-19
Geltrex	Gibco	A1413202
GlutaMAX	Gibco	35050061
Heparin	Sigma	H3149
Human Ultrasensitive Insulin ELISA	ALPCO Diagnostics	80-INSHUU-E01.1
ITS-X	Invitrogen	51500056
KGF	Peprotech	AF-100-19
Knockout DMEM	Gibco	10829018
KnockOut Serum Replacement	Gibco	10828028
L-3,3',5-Triiodothyronine (T3)	EMD Millipore	642245
LDN193189	Stemgent	04-0074
Matrigel Matrix	Corning	354277
MCDB-131	Cellgro	15-100-CV
MEM NEAA	Gibco	11140050
mTeSR 1	StemCell Technologies	5850
N2 Supplement	Life Technologies	17502048
$NaHCO_3$	Sigma	S3817
Noggin Fc Chimera Protein	R&D Systems	3344-NG-050
PdBU	EMD Millipore	524390
Penicillin/Streptomycin	Mediatech	30-002-CI
RPMI	Gibco	11875-093
Sant1	Sigma-Aldrich	S4572
SB431542	Stemgent	04-0010
Smoothened Agonist, SAG	EMD Millipore	566660
StemPro Accutase	Gibco	A1110501
TrypLE	Gibco	12604013
Ultra-Low Attachment Microplates	Corning	3471
Vitamin C	Sigma-Aldrich	A4544
Wnt3a	R&D Systems	5036-WN
XAV 939	Tocris	3748
XXI	EMD Millipore	565790

Y-27632	StemCell Technologies	72302

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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.