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Cover Letter

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September 17th, 2019

Phillip Steindel, PhD
Review Editor, JoVE

Dear Dr. Steindel:

Thanks for inviting us to contribute to *JoVE*.
Attached is our revised manuscript entitled "Efficient and Fast Neural Differentiation through Single Cell Type Culture of Human Embryonic Stem Cells" by Kilsoo Jeon, Kyeyoon Park and Anton M. Jetten.
In the attached rebuttal we have addressed the comments by the Editor and the reviewers.

We believe that our improved protocol to culture and differentiate single cell type hESCs, which has been based on other previously published methods, is fast and scalable and will be suitable for drug screening and stem cell therapy. We think that it will be of interest to many investigators in the field of stem cell research and regenerative medicine and useful in screening assays.

I hope that with the revision we made that the manuscript will be acceptable for JoVE.
I would like to thank you for considering this manuscript for publication in *JoVE.*

Sincerely yours,

Dr. Anton M. Jetten, Ph.D.
Deputy Chief IIDL
Head Cell Biology Section
NIEHS, NIH

TITLE:

Efficient Neural Differentiation using Single-Cell Culture of Human Embryonic Stem Cells

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KEYWORDS:

human embryonic stem cells, single cell culture, differentiation, neural progenitor cells, dopaminergic neurons, astrocytes

SUMMARY:

Presented here is a protocol for the generation of a single-cell culture of human embryonic stem cells and their subsequent differentiation into neural progenitor cells. The protocol is simple, robust, scalable, and suitable for drug screening and regenerative medicine applications.

ABSTRACT:

In vitro differentiation of human embryonic stem cells (hESCs) has transformed the ability to study human development on both biological and molecular levels and provided cells for use in regenerative applications. Standard approaches for hESC culture using colony type culture to maintain undifferentiated hESCs and embryoid body (EB) and rosette formation for differentiation into different germ layers are inefficient and time-consuming. Presented here is a single-cell culture method using hESCs instead of a colony-type culture. This method allows maintenance of the characteristic features of undifferentiated hESCs, including expression of hESC markers at levels comparable to colony type hESCs. In addition, the protocol presents an efficient method for neural progenitor cell (NPC) generation from single-cell type hESCs that produces NPCs within 1 week. These cells highly express several NPC marker genes and can differentiate into various neural cell types, including dopaminergic neurons and astrocytes. This single-cell culture system for hESCs will be useful in investigating the molecular mechanisms of these processes, studies of certain diseases, and drug discovery screens.

INTRODUCTION:

Human embryonic stem cells (hESCs) have the potential to differentiate into the three primary germ layers, which then differentiate into various multipotent progenitor cell lineages. These lineages subsequently give rise to all cell types in the human body. In vitro hESC culture systems have transformed the ability to study human embryonic development and have served as a valuable tool for obtaining new insights into how these processes are regulated at the biological and molecular levels. Similarly, studies of induced pluripotent stem cells (iPSCs) generated from reprogramming somatic cells isolated from human patients provide novel insights into various diseases. In addition, progenitor and differentiated cells derived from hESCs can be useful for research involving stem cell therapy and drug screening[1-4].

hESCs can be induced to differentiate into neural progenitor cells (NPCs), which are multipotential cells with an extensive self-renewal capacity. Subsequently, these cells can be differentiated into neurons, astrocytes, and oligodendrocytes[5,6]. NPCs also offer a cellular system for in vitro studies of neurodevelopmental biology and various neurological diseases. However, current colony type culture methods involving hESCs and their differentiation into NPCs are inefficient and often involve coculture as well as embryoid body (EB) and rosette formation[5,7-9]. These protocols exhibit lower survival rates and spontaneous differentiation and are more time-consuming.

Presented here is an improved and robust culture system that is easily scalable and uses high density single-cell type culture of hESCs[10]. The inclusion of Roh-kinase (ROCK) inhibitor contributed to significantly enhanced survival efficiency during single cell type culture of hESC[10-14]. In this culture system, hESCs can be easily maintained and expanded. In addition, the protocol presents an efficient method to generate NPCs from single-cell type culture of hESCs, which allows the production of highly pure NPCs. Inhibition of BMP/TGFβ/activin signaling pathways with ALK inhibitors efficiently induce differentiation of single-cell type hESCs into NPCs[15,16], which then can be induced to differentiate into functional neural lineages, such as dopaminergic neurons and astrocytes.

In summary, the single-cell type culture protocol using hESCs offers an attractive model to study the differentiation of these cells into various lineages, including NPCs. This protocol is easily scalable and therefore suitable for generating cells for research involving regenerative therapy and drug screening.

PROTOCOL:

1. Preparation of hESC-qualified basement membrane matrix-coated plates

1.1. Slowly thaw the hESC-qualified basement membrane matrix (see **Table of Materials**) solution at 4 °C for at least 2–3 h or overnight to avoid formation of a gel.

1.2. To prepare basement membrane matrix-coated plates, dilute matrix in cold DMEM/F12 to a 2% final concentration. Mix well and coat each well of a 6 well plate with 1 mL of the diluted matrix solution.

1.3. Incubate the basement membrane matrix-coated plates at room temperature (RT) for at least 3 h or at 4 °C overnight.

NOTE: Plates with basement membrane matrix can be stored at 4 °C for 1 week before the matrix solution is removed and plates are used.

2. Adaptation of colony type hESCs to single-cell hESC culture

2.1. To passage the feeder-free cultures of colony type H9 (WA09) hESCs grown on basement membrane matrix, aspirate the medium from the wells (**Figure 1A**)[9].

2.2. Wash 1x with 1 mL of DPBS. Add 1 mL of dispase solution (1 U/mL) per well and incubate at 37 °C for 20 min.

2.3. Remove the dispase, wash the cells 1x gently with 2 mL of DMEM/F12, remove the medium, and add 2 mL of DMEM/F12 to each plate.

2.4. Gently detach colonies by gently pipetting up and down and transfer to a 15 mL tube.

2.5. Centrifuge the pellets for 2 min at 370 x *g* and aspirate the medium.

2.6. To dissociate the cell pellets into single cells, add 2 mL of cell detachment solution (1x concentration, see **Table of Materials**) and incubate at 37 °C for 10 min.

2.7. Centrifuge cells for 2 min at 370 x *g* and remove the detachment solution.

2.8. Add fresh mTeSR1 human ESC medium and dissociate the cells into single cells by gentle pipetting up and down.

2.9. To adapt colony type hESCs to a single-cell type culture, plate approximately 1.5–2.0 x 10^6 hESCs into each well of the basement membrane matrix-coated 6 well plate in 2 mL of mTeSR1 containing 10 µM ROCK inhibitor for 24 h (**Figure 1A**).

2.10. After 24 h, replace the hESC medium with fresh mTeSR1 without ROCK inhibitor and allow the hESCs to grow as a single-cell type for 3 days. Change the medium daily.

2.11. On day 4, when cultures reach nearly 100% confluency, dissociate cells in detachment solution, then replate as described in step 2.6.

NOTE: The ROCK inhibitor improves cell survival during the initial 24 h of single-cell type hESC culture.

3. Embryoid body formation and differentiation into three germ layers (Figure 2)

3.1. To form EBs, first resuspend cells in 3 mL of mTeSR1 medium with 10 µM ROCK inhibitor during the first 24 h, then incubate overnight into 60 mm low attachment dishes in a 37 °C incubator to allow aggregation.

3.2. After 24 h, the small EBs are transferred to a 15 mL tube. Let the EBs settle to the bottom of the tube and gently remove the medium with a pipette. Transfer EBs into EB medium (knockout-DMEM supplemented with 20% knockout serum replacement, 1x glutamine supplement [see **Table of Materials]**, 1% NEAA [non-essential amino acids; see **Table of Materials**], and 0.2% β-mercaptoethanol) and allow them to expand in low attachment dishes for 7 days. The medium can be changed every other day as described above (**Figure 2A**).

3.3. On day 7, collect the EBs from the dishes and transfer them into a 15 mL tube. Gently remove the medium with a pipette and transfer the EBs to a basement membrane matrix-coated 6 well plate.

3.4. Allow the EBs to attach to the plate and incubate for 12 days in EB medium, during which they will differentiate into the three germ layers (**Figure 2B**). Change the medium every other day.

NOTE: During aggregation of cells, the low attachment dish helps avoid EB attachment.

4. Differentiation of single-cell type hESCs into NPCs (Figure 3)

4.1. To induce NPC differentiation, dissociate single-cell type hESCs with 1 mL of detachment solution (1x) and incubate for 10 min at 37 °C.

4.2. Centrifuge the cells for 2 min at 370 x *g* and remove the detachment solution supernatant. Add 1 mL of DMEM/F12 and resuspend cells by gentle pipetting.

4.3. Plate cells on a basement membrane matrix-coated 6 well plate at a density of 2 x 10^5 cells/well in 2 mL of mTeSR1 containing 10 µM ROCK inhibitor.

4.4. After 24 h, replace the culture medium with neural induction medium (DMEM with 1% B27 minus vitamin A) supplemented with 1 µM dorsomorphin and 5 µM SB431542.

4.5. Change the medium every other day during the first 4 days of neural induction, then every day until reaching confluence at day 7 (**Figure 3B**).

NOTE: (1) Dorsomorphin inhibits the BMP pathway by targeting ALK2,3,6 receptors and also inhibits AMPK; SB431542 is an inhibitor of the TGFβ/Activin pathway by targeting ALK5,7. (2) The same protocol was tested with another ESC line (WA01), which yielded similar results as WA09[16]. (3) We have generally used Matrigel for the basement membrane matrix; however, cells can be cultured on Geltrex and then differentiated into NPCs with very similar results as indicated by the expression of several NPC markers (**Figure 4D,E**). Handle Geltrex in the same manner as Matrigel (see section 1).

5. NPC Expansion and Cryopreservation

5.1. After 7 days of neural induction, dissociate cells with 1 mL of detachment solution (1x) and incubate for 10 min at 37 °C.

5.2. Centrifuge the cells for 2 min at 370 x *g* and remove the detachment solution supernatant. Add 1 mL of NPC expansion medium (see **Table of Materials**) and resuspend cells by gentle pipetting.

5.3. Plate 1 x 10^5 cells in 2 mL of NPC expansion medium on basement membrane matrix-coated 6 well plates.

5.4. Passage NPCs multiple times, as necessary, when cultures reach nearly 90% confluency. During the first 3–4 passages, add 10 µM ROCK inhibitor during the initial 24 h to prevent cell death. After these passages, cells can be passaged and cultured without ROCK inhibitor. Change the medium every other day during the expansion.

5.5. Cryopreserve NPCs when cultures reach confluency.

5.5.1. For cryopreservation, dissociate cells in 1 mL of detachment solution (1x) for 5 min at 37 °C, then centrifuge suspension for 2 min at 370 x *g* to remove the detachment solution.

5.5.2. Add cell freezing solution (see **Table of Materials**) to dissociated NPCs at 1 x 10^6 cells/mL and distribute 1 mL aliquots to cryovials.

5.5.3. Place the cryovials into freezing container and store them overnight in a -80 °C freezer.

5.5.4. Store the cryovials in the liquid nitrogen.

6. Preparation of poly-L-ornithine (PLO) and laminin coated plates

6.1. To prepare PLO/laminin coated plates, dilute the PLO stock solution into PBS or water to a final concentration of 10 µg/mL. Mix well and coat each well of a 6 well plate with 1 mL of diluted PLO solution.

6.2. Incubate for at least 2 h at 37 °C or overnight at 4 °C.

6.3. Wash each well with PBS.

6.4. Dilute laminin stock solution in cold PBS or water at 10 µg/mL. Mix the laminin solution well. Remove the PBS and immediately coat each well with 1 mL of the laminin solution. Keep plates at 4 °C and use within 1 week. Wash with PBS and immediately add culture medium.

NOTE: Do not let the PLO/laminin coated plates dry out.

7. Differentiation of hESC-derived NPCs into dopaminergic neurons (Figure 6A)

7.1. Plate NPCs in PLO/laminin-coated dishes in NPC expansion medium at a density of approximately 50%.

7.2. After 24 h, change the medium to DA1 medium (neurobasal medium containing 2 mM glutamine supplement, 1x NEAA, 1x B27, 200 ng/mL SHH, and 100 ng/mL FGF8) and change the medium every other day.

7.3. Passage cultures when they reach confluency. Dissociate cells with 1 mL of detachment solution (1x) and incubate for 10 min at 37 °C.

7.4. Centrifuge the cells for 2 min at 370 x *g* and remove the detachment solution supernatant. Add 1 mL of DA1 medium and resuspend the cells by gentle pipetting.

7.5. Plate 1 x 10^5 cells in 2 mL of DA1 medium on PLO/laminin-coated 6 well plates.

7.6. After 10 days, change to DA2 medium (neurobasal medium containing 2 mM glutamine supplement, 1x NEAA, 1x B27, 200 µM ascorbic acid, 20 ng/mL BDNF, and 20 ng/mL GNDF) and change medium every other day for an additional 20 days (**Figure 6A**).

NOTE: Add fresh ascorbic acid daily to the DA2 medium. Within the first 14 days, the differentiated cells should be passed when they reach 90% confluency, but no longer after that.

8. Differentiation of ESC-derived NPCs into astrocytes

8.1. Plate neural progenitor cells on PLO/laminin-coated plates in NPC expansion medium at a density of approximately 50%.

8.2. After 24 h, change the medium to astrocyte medium (DMEM/F12 medium including 1:100 N2, 1:100 B27, 200 ng/mL IGF, 10 ng/mL activin A, 10 ng/mL heregulin β-1, and 8 ng/mL bFGF) and change the medium every other day.

8.3. Passage cultures when they reach confluency. Dissociate cells with 1 mL of detachment solution (1x) and incubate for 10 min at 37 °C.

8.4. Centrifuge the cells for 2 min at 370 x *g* and remove the detachment solution supernatant. Add 1 mL of astrocyte medium and resuspend the cells by gentle pipetting.

8.5. Plate 1 x 10^5 cells in 2 mL of astrocyte medium on PLO/laminin-coated 6 well dishes.

8.6. Allow differentiation to continue for a total of 5–6 weeks (**Figure 6B**).

9. Immunofluorescence staining

9.1. Culture cells in 35 mm µ-dishes as described above for each cell type. Aspirate the medium and add 1 mL of 4% PFA solution per dish and incubate for 20 min at RT.

9.2. Wash 2x for 5 min with PBS.

NOTE: Once the cells are fixed, the assay plates can be sealed with parafilm and stored at 4 °C. Stain with an antibody within 1 week after fixation.

9.3. Add 300 µL of blocking solution (goat or donkey serum in PBS) per dish and incubate at RT for 30–60 min.

9.4. Wash the dish 2x with PBS for 5 min.

9.5. Add 300 µL of primary antibody (**Table 1**) solution (diluted in blocking solution) and incubate at RT for 1.5 h or overnight at 4 °C.

9.6. Wash 2x with PBS for 10 min.

9.7. Add 300 µL of fluorescent-conjugated secondary antibody (**Table 1**) in blocking solution and incubate in the dark for 1 h at RT.

9.8. Wash the cells 2x for 10 min with PBS.

9.9. Add Hoechst staining solution (1 µM final concentration in PBS) to stain nuclei. Incubate the cells in the dark for 5 min at RT.

9.10. Wash cells 1x with PBS for 5 min and then add 500 µL of PBS. Keep dishes in the dark, then observe fluorescence with a confocal microscope (**Figure 2B**, **Figure 5C**, **Figure 6A**, and **Figure 7B**).

REPRESENTATIVE RESULTS:

Presented here is an improved protocol for the maintenance and expansion of single-cell type culture of hESCs and their efficient differentiation into neural progenitor cells, which

subsequently differentiates into various downstream neural lineages, including dopaminergic neurons and astrocytes.

Representative phase contrast images show cell morphology at different steps during the adaptation of colony type hESCs to the single-cell type culture (**Figure 1A**). Through the single-cell culture condition, it was found that the adapted hESCs were able to be maintained at high density, then easily and efficiently subcultured when reaching confluency (**Figure 1A,B**). These cells retained the cell cycle characteristics (i.e., a short G1 phase and high proportion of cells in S phase) typical of colony type hESCs (**Figure 1C,D**). They also expressed the ESC markers (i.e., OCT4, TRA 1-81, SOX2, and NANOG) at levels comparable to those of colony type hESCs as indicated by QRT-PCR and immunostaining analysis (**Figure 7**, **Table 2**). Moreover, it was shown that single-cell hESCs were able to form embryoid bodies containing cells from all three germ layers: endoderm (SOX17 expression), mesoderm (SMA expression) and ectoderm (Tuj-1 expression) (**Figure 2**).

Next, it was demonstrated that single-cell type hESCs efficiently differentiated into neural progenitor cells using an NPC protocol (**Figure 3A**), as indicated by the loss of typical hESC morphology and appearance of NPC morphology (**Figure 3B**)[16]. NPC differentiation was supported by the increased expression of the signature NPC markers (i.e., SOX1, OTX2, ZIC1, and OTX1; **Figure 5A**, **Table 2**) and confirmed by immunostaining and FACS analysis. The same analysis also showed that more than 90% of the cells stained positive for SOX1, PAX6, and NCAM protein (**Figure 5B,C**). To examine the ability of single-cell hESC-derived NPCs to differentiate into various downstream neural lineages, the differentiation of these cells into dopaminergic neurons and astrocytes was examined. As shown in **Figure 6A,B**, single-cell hESC-derived NPCs were able to differentiate into dopaminergic neurons and astrocytes, as indicated by the appearance of characteristic morphologies and expression of lineage-specific dopaminergic markers (i.e., TH; **Figure 6A**) and astrocyte markers (i.e., GFAP and S100B; **Figure 6B**).

FIGURE LEGENDS:

Figure 1: Adaptation of colony type hESCs to single-cell type culture. (**A**) Representative phase contrast images of single cell cultures of H9 hESCs at different times after plating on 2% basement membrane matrix-coated dishes. Low (left) and high (right) magnification. Top panel: representative image of colony type hESCs. Other panels show representative images of cultures at different times during the adaptation to single-cell type hESCs. Scale bar = 200 µm. (**B**) Growth curves of H9 hESCs were monitored in 2% basement membrane matrix-coated plates with 10 µM ROCK inhibitor for the first 24 h during the single-cell culture condition. (**C,D**) Cell cycle analysis of colony type (**C**) and single-cell type (**D**) H9 hESCs by flow cytometry.

Figure 2: In vitro differentiation of adapted single-cell type hESCs into three germ layers. (**A**) Representative phase images of embryonic bodies (EB) derived from single-cell type of hESCs. Scale bar = 100 µm. (**B**) Immunofluorescent images of differentiated hESCs analyzed for the

expression of the three different germ layer markers: SOX17 (endoderm), SMA (mesoderm), and Tuj-1 (ectoderm). Nuclei were stained with DAPI. Scale bar = 50 µm.

Figure 3: Differentiation of single-cell type hESCs into neural progenitor cells by direct differentiation. (**A**) Schematic of the differentiation protocol of hESCs into neural progenitor cells (NPCs). hESCs were treated with dorsomorphin (DMH) and SB431542 (SB) 1 day after plating. (**B**) Representative phase contrast images of cell morphology during neural differentiation. Scale bar = 200 µm.

Figure 4: hESC culture on different basement membrane matrix products. (**A**) hESCs cultured on Matrigel or Geltrex exhibited an ability to grow and differentiate into NPCs that was similar to the single cell-culture. Cells were stained with a NESTIN antibody. Nuclei were stained with DAPI. Scale bar = 50 µm. (**B**) hESCs cultured on Matrigel or Geltrex showed similar potential to differentiate into NPCs as indicated by the percentage of NESTIN- and PAX6-positive cells. Cells were analyzed by flow cytometry at day 7 of NPC differentiation.

Figure 5: Expression of NPC markers. (**A**) After 7 days of neural differentiation, expression of NPC marker genes (i.e., OTX1, OTX2, SOX1, and ZIC1) was analyzed by QRT-PCR. Values were normalized to GAPDH and calculated relative to the values of hESCs ($p < 0.05$). (**B**) The percentage of SOX1-, NESTIN-, SOX2-, and NCAM-positive cells was determined by flow cytometry at day 7 of NPC differentiation. (**C**) At day 7 NPC differentiation, cells were stained with antibodies against the neural markers SOX1, NESTIN, NCAM, OTX2, and PAX6. Nuclei were stained with DAPI. Scale bar = 100 µm.

Figure 6: Dopaminergic neuron and astrocyte differentiation of NPCs derived from single-cell type hESCs. (**A**) Representative phase contrast image of dopaminergic neurons (top panel). Scale bar = 100 µm. Differentiated cells were stained with antibodies against the dopaminergic neuron marker TH (tyrosine hydroxylase) as indicated. Nuclei were stained with DAPI. Scale bar = 50 µm. (**B**) Representative phase contrast image of astrocytes (top panel). Scale bar = 100 µm. Differentiated cells were stained with antibodies against the astrocyte marker GFAP (glial fibrillary acidic protein) and S100-B (S100 calcium binding protein B), as indicated. Nuclei were stained with DAPI. Scale bar = 50 µm.

Figure 7: Characterization of single cell type hESCs. (**A**) hESCs adapted to single-cell type culture were analyzed for the expression of ESC markers (i.e., OCT4, NANOG, and SOX2) by QRT-PCR. Values were normalized to GAPDH ($p < 0.05$). (**B**) Immunofluorescent images of hESCs stained for the expression of the pluripotency markers OCT4, TRA-1-81, and SSEA-1. Nuclei were stained with DAPI. Scale bar = 50 µm.

Table 1: Antibodies used in immunocytochemistry and FACS analysis.

Table 2: List of primers used in QRT-PCR analysis.

DISCUSSION:

Scalable and efficient methods for the differentiation of hESCs into various lineages and the generation of sufficient numbers of differentiated cells are important criteria for drug screening and stem cell therapy. Various single-cell passing methods have been published, in which cells are cultured in the presence of ROCK inhibitor or other small molecules to improve survival, but the final products of these culture methods are colony type hESCs[17-21]. The single-cell ESC protocol, which is partially based on previously published methods[19-22], successfully generates and maintains single cell-type hESC cultures and prevents colony type hESC culture. It includes high density single cell plating, multicellular association, monolayer growth, and efficient subculture (**Figure 1**). The latter was achieved by the addition of ROCK inhibitor during the initial 24 h of single-cell type culture of hESCs, which improves cell survival[17-21]. This protocol is more easily scalable and allows expansion of these cells for therapeutic applications in drug screening and stem cell.

It is further demonstrated that single-cell type hESCs can efficiently differentiate to the NPC lineage (**Figure 3**) without use of an intermediate stage, such as EB and rosette formation[23-25]. High neural conversion from single-cell type hESCs was achieved through the inhibition of BMP/TGFβ/activin signaling pathways by treatment with the ALK inhibitors, dorsomorphin, and SB431542[15,16,26]. With this protocol, the adapted single-cell type hESCs can efficiently differentiate into NPCs without the need for EB and rosette formation (**Figure 5**) and or be induced to differentiate into dopaminergic neurons and astrocytes (**Figure 6**).

In summary, single-cell type culture of hESCs provides a rapid and efficient system to study the molecular mechanisms that regulate multistep differentiation to various lineages. Specifically, this protocol utilized NPCs and described their subsequent differentiation into additional neural lineages, such as astrocytes and dopaminergic neurons. The protocol provides a platform for simple, robust, and scalable production of progenitor and differentiated cells that can be suitable for basic studies, drug screening, and applications in regenerative medicine.

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DISCLOSURES:
The authors declare no conflicts of interest.

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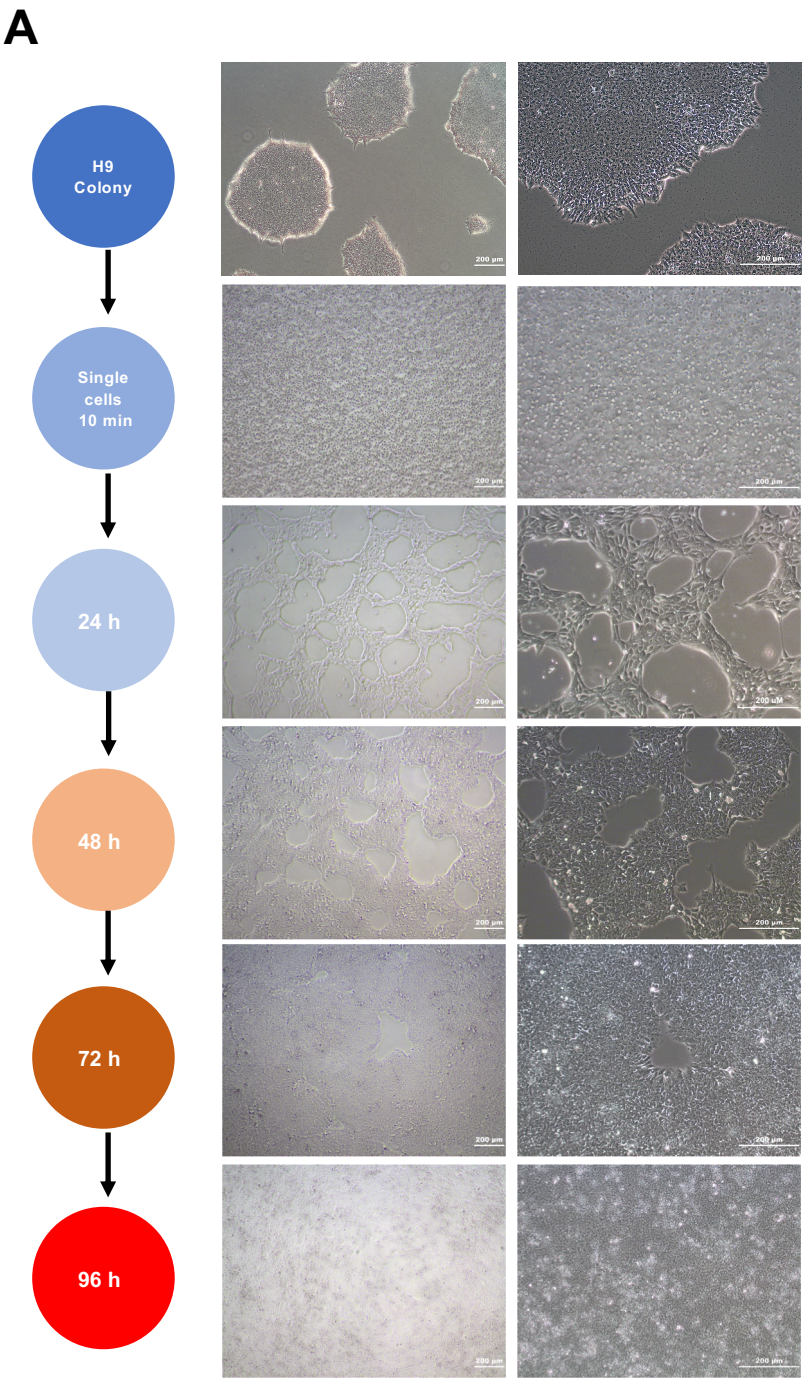
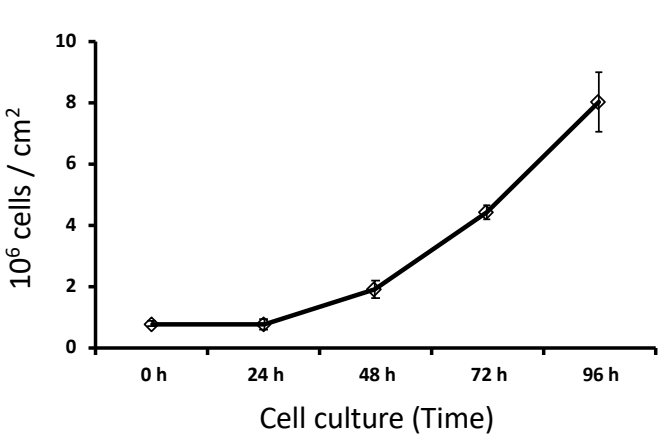
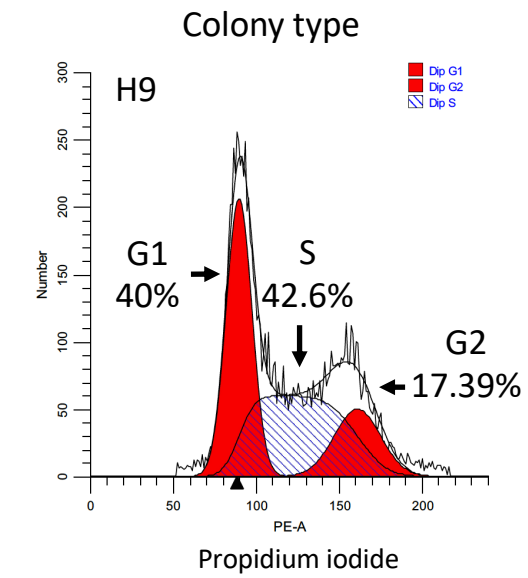
Figure 1







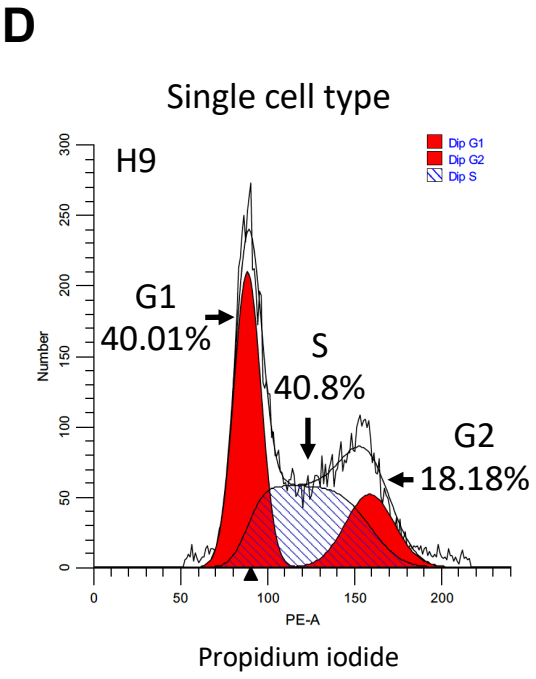
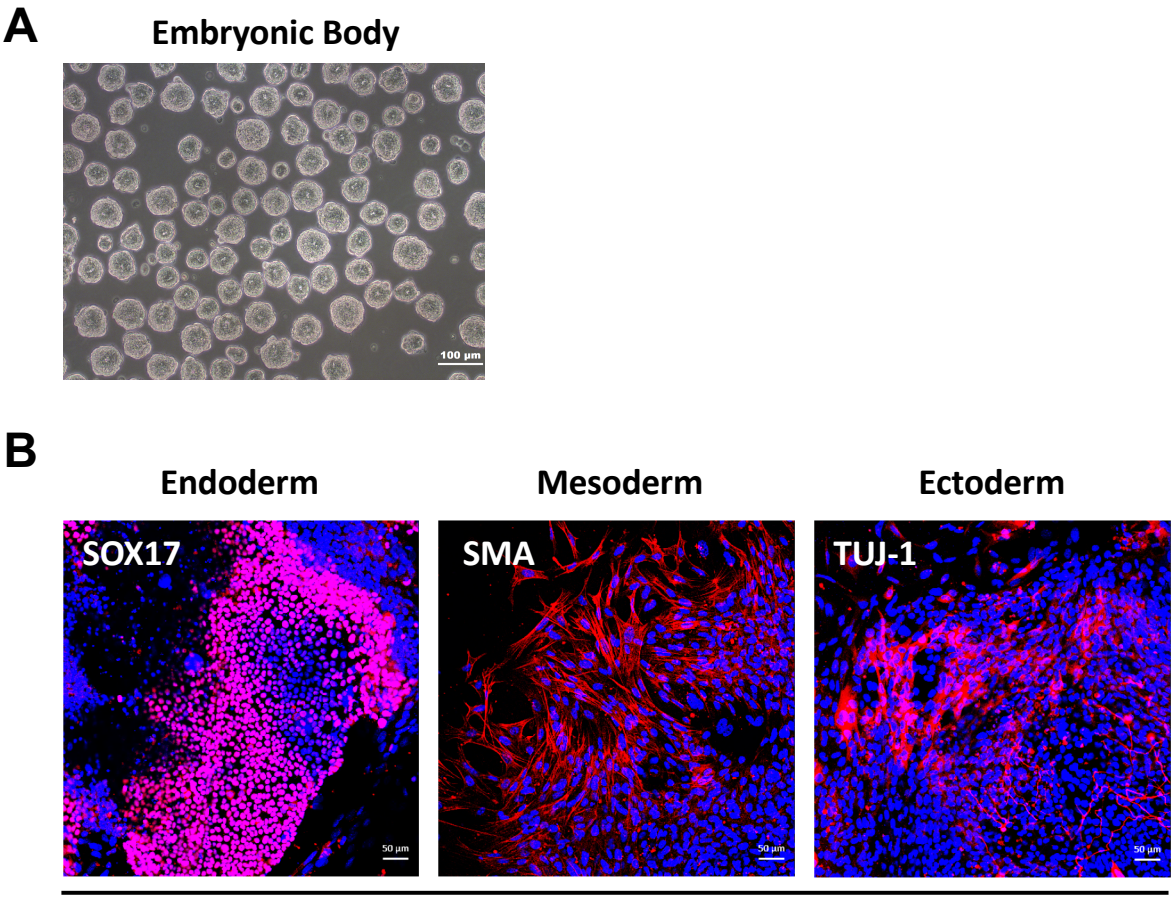


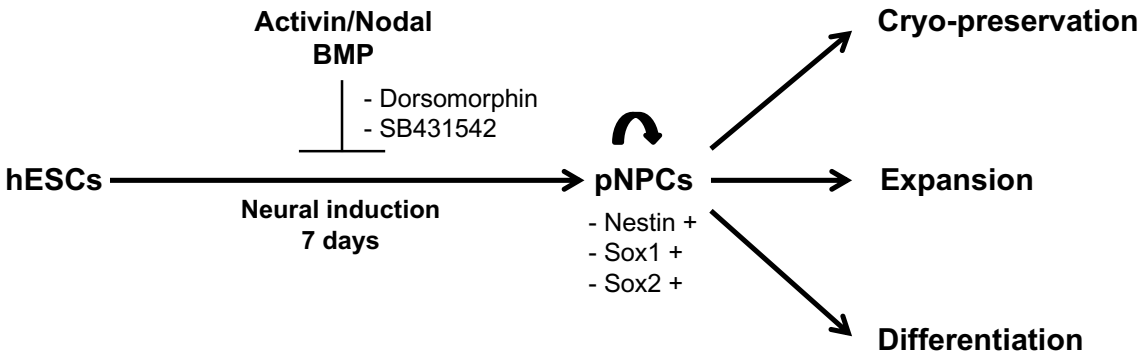
Figure 2



Three germ layers derived from Single cell

Figure 3

A



B

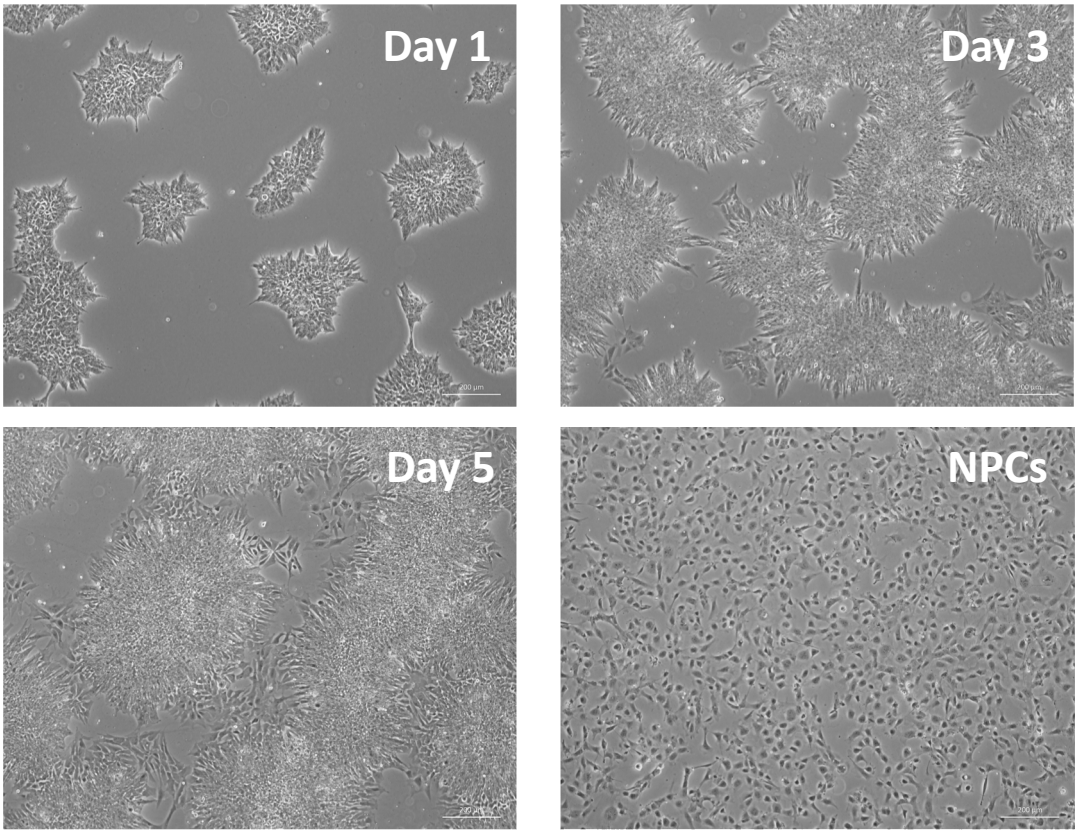


Figure 4

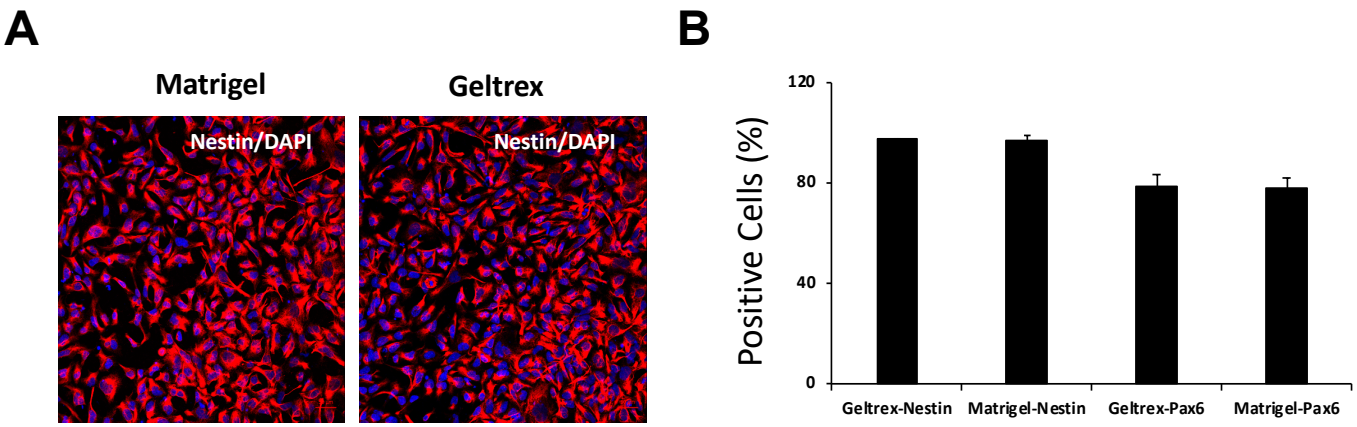


Figure 5

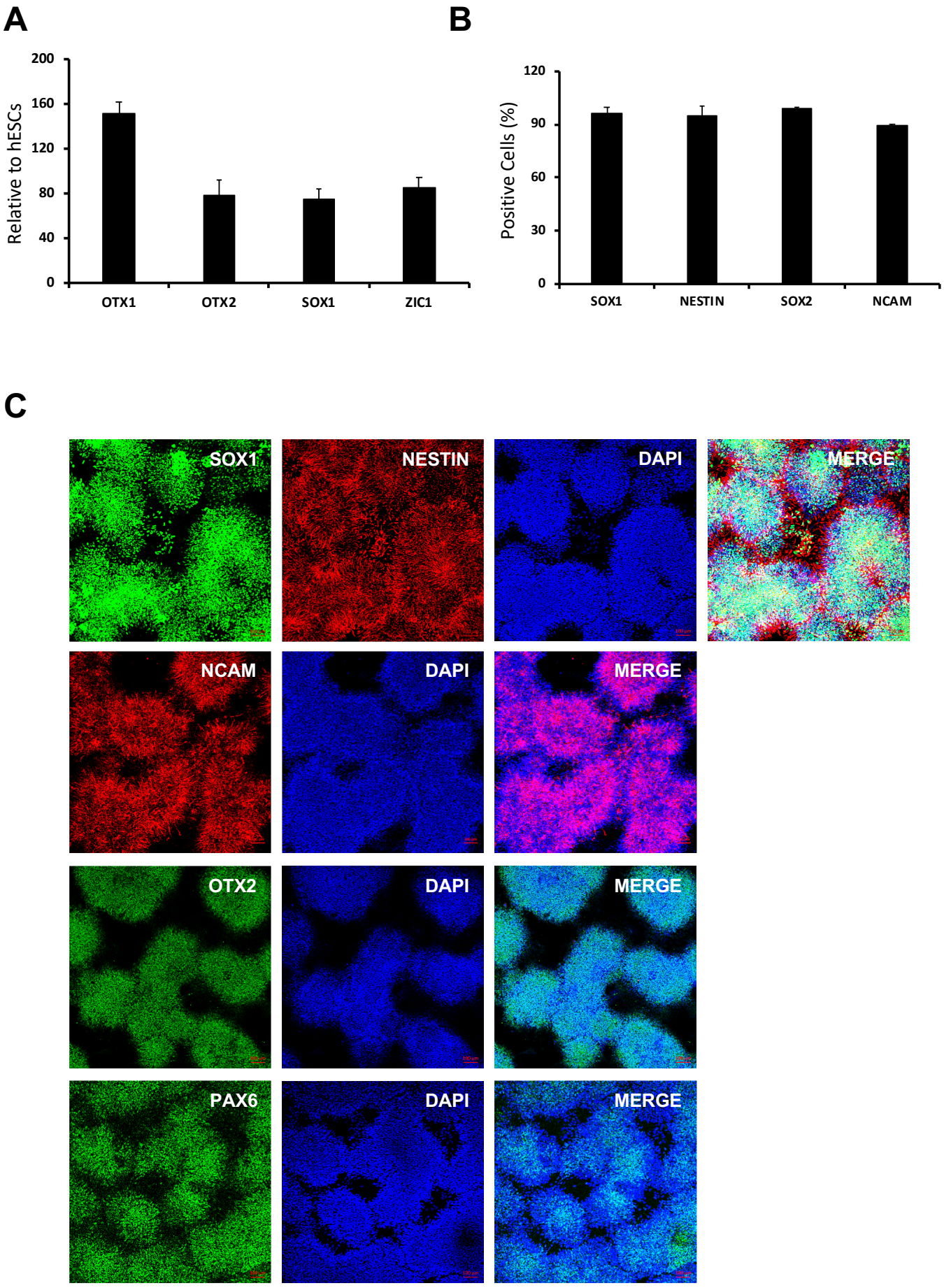


Figure 6

Click here to access/download;Figure;Figure 6.pdf

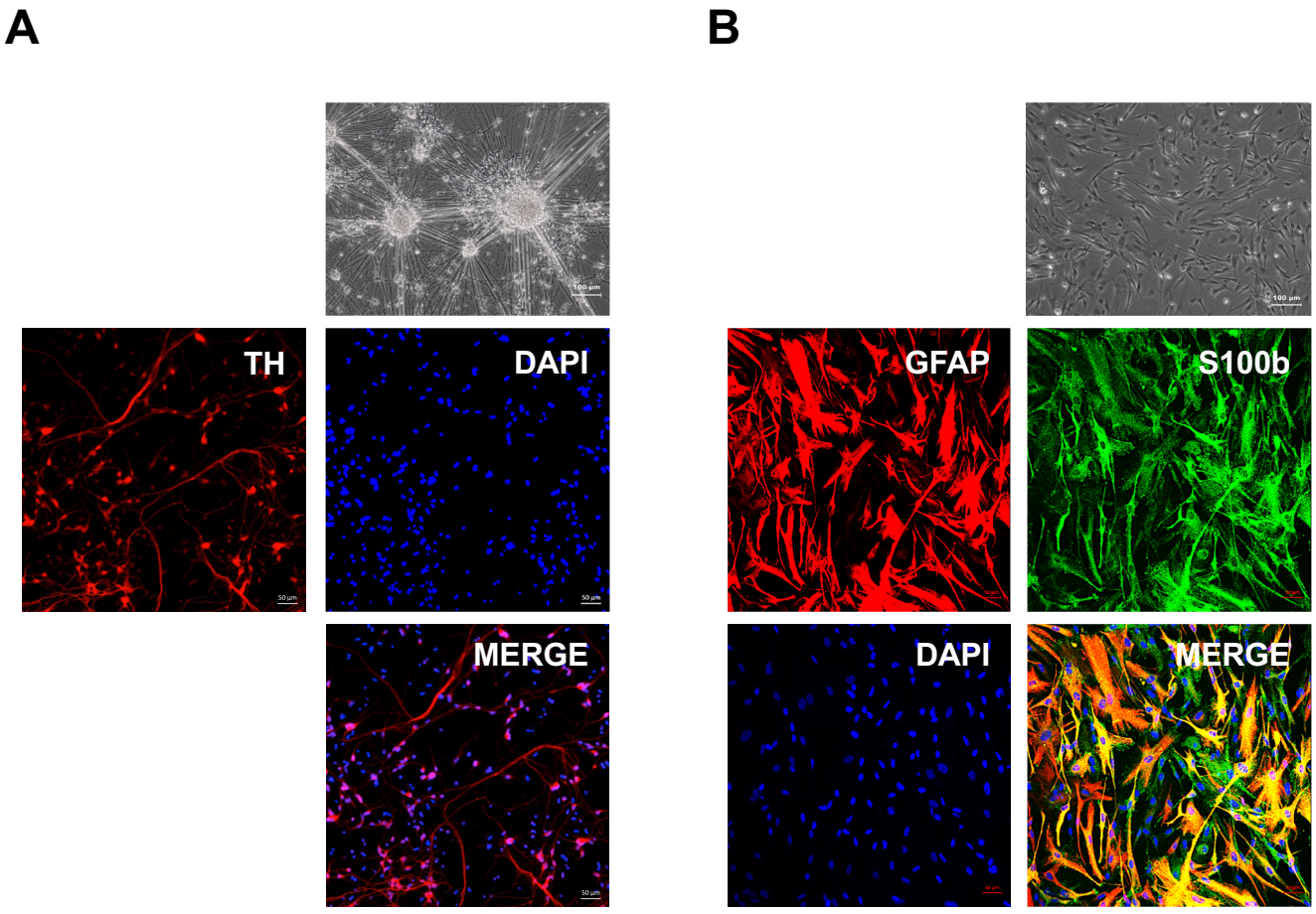


Figure 7

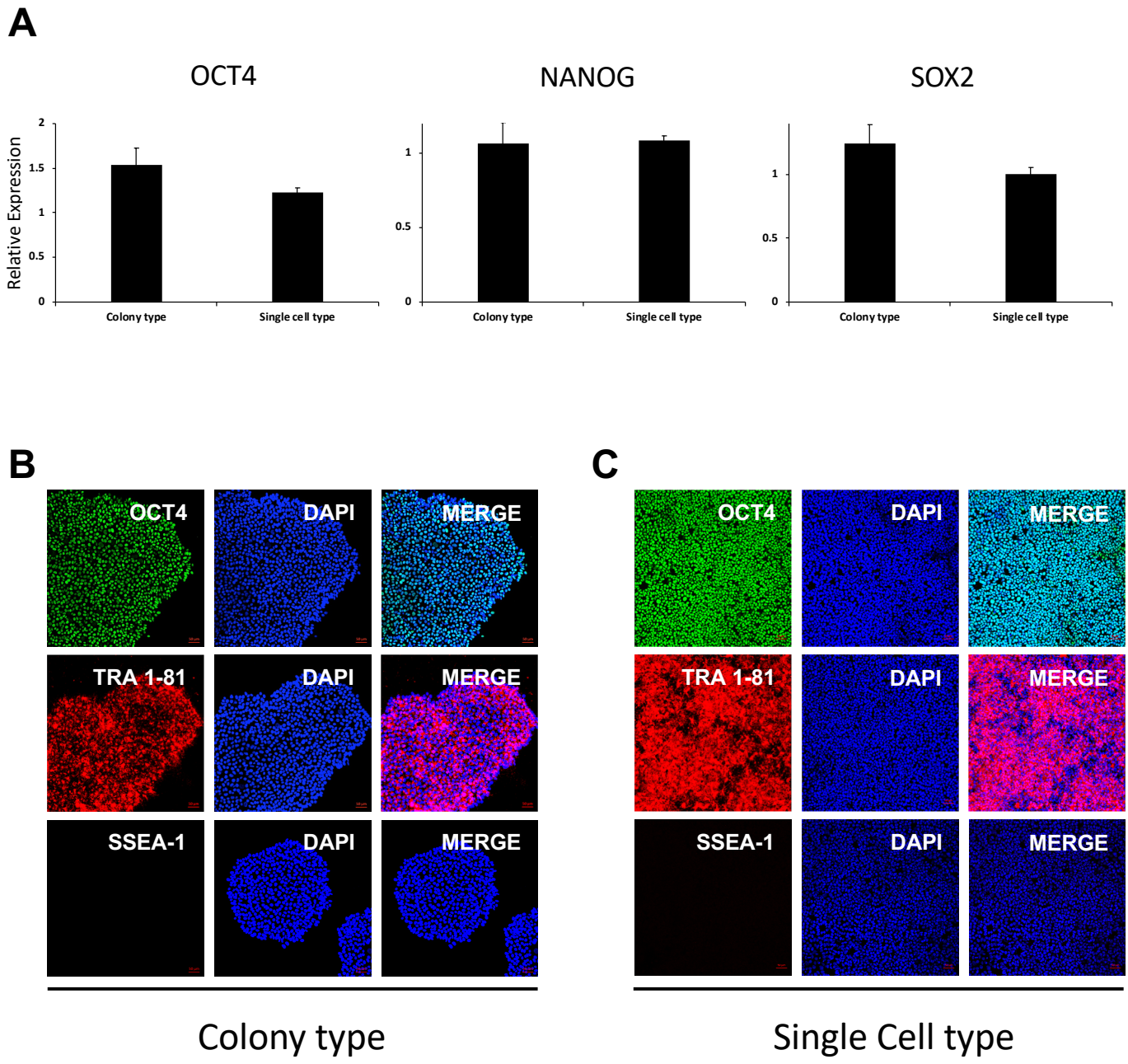


Table 1

Primary Antibodies	Species	Dilution	Catalog Number	Company
OCT4	Mouse	1:1000	sc-5279	Santa Cruz
TRA 1-81	Mouse	1:500	sc-21706	Santa Cruz
SSEA-1	Mouse	1:500	sc-21702	Santa Cruz
SOX1	Goat	1:500	AF-3369	R&D
SOX2	Rabbit	1:1000	sc-20088	Santa Cruz
SMA	Rabbit	1:500	ab-5694	Abcam
Tuj1	Mouse	1:1000	T8578	Sigma
NESTIN	Mouse	1:1000	ab-22035	Abcam
OTX2	Mouse	1:250	ab-21990	Abcam
hNCAM	Mouse	1:200	sc-106	Santa Cruz
hPAX6	Mouse	1:250	561664	BD
TH	Mouse	1:500	T1299	Sigma
S100b	Mouse	1:250	ab4066	Abcam
GFAP	Rabbit	1:1000	ab7260	Abcam
Secondary Antibodies				
Anti-mouse	Goat	1:1000	AF 488 or 647	Life Technologies
Anti-rabbit	Goat	1:1000	AF 488 or 647	Life Technologies

Table 2

Primer name	Primer sequence
OCT4	F: GGAAGGTATTCAGCCAAACG
	R: CTCCAGGTTGCCTCTCACTC
NANOG	F: GGTTCCAGAACCAGAGAATGA
	R: ATTGGAAGGTTCCCAGTCG
SOX1	F: CCTTAGGTTTCCCCTCGCTTT
	R: CAGGCTGAATTCGGTTCTCATT
OTX1	F: AAGATCAACCTGCCGGAGTCT
	R: CGTGAATTGGCCACTGCTTT
OTX2	F: TGGAAGCACTGTTTGCCAAG
	R: TAAACCATACCTGCACCCTCG
ZIC1	F: AACCCCAAAAAGTCGTGCAAC
	R: TCCTCCCAGAAGCAGATGTGA
GAPDH	F: CCCATCACCATCTTCCAGGAG
	R: CTTCTCCATGGTGGTGAAGACG

Name of Material/Equipment	Company	Catalog Number	Comments/Description
35 mm μ-dishes	ibidi	81156	Cell culture dish
6-well plates	Corning	3516	
Accutase	Innovative Cell Technologies	AT104-500	Cell detachment solution
Activin A	R&D system	338-AC-050	
Ascorbic Acid	Sigma Aldrich	A4403	
B27 supplement	Thermo Fisher	17504044	
B27 supplement (-Vit A)	Thermo Fisher	12587010	
BDNF	Applied Biological Materials	Z100065	
bFGF	Peprotech	100-18C	
Centrifuge	DAMON/ICE	428-6759	
CO2 incubator	Thermo Fisher	4110	
Corning hESC-qulified Matrix (Magrigel)	Corning	354277	Basement membrane matrix (
Cryostor CS 10	Stemcell Technologies	7930	Cell freezing solution
Dispase	Stemcell Technologies	7923	
DMEM	Thermo Fisher	10569-010	
DMEM/F12	Thermo Fisher	10565-018	
Dorsomorphin	Tocris	3093	
EGF	Peprotech	AF-100-16A	
Fetal Bovine Serum	Fisher Scientific	SH3007003HI	
FGF8	Applied Biological Materials	Z101705	
GDNF	Applied Biological Materials	Z101057	
Geltrex matrix	Thermo Fisher	A1569601	Basement membrane matrix
GlutaMax	Thermo Fisher	35050061	Glutamine supplement, 100X
H9 (WA09) human embryonic stem cell line	WiCell	WA09	
Heregulin β-1	Peprotech	100-3	
IGF	Peprotech	100-11	
Knockout DMEM	Thermo Fisher	10829018	
Knockout Serum Replacement	Thermo Fisher	10828028	
Laminin	Sigma Aldrich	L2020	
mTeSR1	Stemcell Technologies	85850	hESC culture medium

N2 supplement	Thermo Fisher	17502001	
NEAA	Thermo Fisher	11140050	
Neurobasal	Thermo Fisher	21103049	
Poly-L-ornithine	Sigma Aldrich	P3655	
ROCK inhibitor	Tocris	1254	
SB431542	Tocris	1614	
SHH	Applied Biological Materials	Z200617	
Stemdiff Neural Progenitor medium	Stemcell Technologies	5833	NPC expansion medium

used for most of the protocol here)

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Author(s):	Kilsoo Jeon, Kyeyoon Park, and Anton M. Jetten

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Editorial comments:

General:

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

Response: We have confirmed this in the revised manuscript.

2. Please ensure that the manuscript is formatted according to JoVE guidel ines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

Response: We have corrected this in the revised manuscript.

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For example: Matrigel, Accutase, GlutaMax, StemDiff

Response: We have corrected this in the revised manuscript.

Protocol:

1. Please ensure each step/substep is written in the imperative.

Response: We have confirmed this in the revised manuscript.

2. Please express centrifuge speeds in x g instead of rpm.

Response: We have corrected this in the revised manuscript.

3. 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.

Response: We have revised the manuscript accordingly.

Specific Protocol steps:

1. 2: Where do the hESCs come from? If they come directly from a clinical source, please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

Response: We have corrected this in the revised manuscript.

2. 2.1: Please include a reference for growing colony-type hESCs.

Response: We have added ref in the revised manuscript.

Figures:

1. Please remove 'Figure. 1' etc. from the Figures themselves.

Response: We have removed “Figs” in the revised manuscript.

2. Figure 1: Please use ‘h’, not ‘hr’. Please also include spaces between numbers and their corresponding units.

Response: We have changed this in the revised manuscript.

Discussion:

1. Discussion: 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 modifications and troubleshooting of the technique

c) Any limitations of the technique

Response: We have revised the Discussion in revised manuscript

References:

1. Please do not abbreviate journal titles.

Table of Materials:

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

Response: We have corrected this in the revised manuscript.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The Invited MS JoVE60571 aims to describe in details some of the methods used in the previous paper doi: 10.1002/stem.2941. Epub 2018 Dec 2. (Stem Cells).

Two main culture systems used in this publications are described in details: 1) human embryonic culture in feeder-free conditions starting from single cells and 2) neural induction of hESCs. Additionally, the MS contains 3) a sub-protocol for spontaneous differentiation of hESC via EBs, 4) a sub-protocol of proliferation of the neuroepithelial/neural progenitor cells, as well as the differentiation of neural progenitor cells toward 5) dopaminergic neurons and 6) astrocytes. As the title and the introduction underline, the main protocol to be analyzed and detailed is the neural induction, which aims to be efficient and fast.

The protocol described here is derived (closely) from the protocol of Chambers (2009) (ref 15), that was intensely used in the field and it was shown during the last 10 years to be fast and efficient, being cited by over 100 publications.

The protocols using neural induction by dual inhibition of SMAD signaling starting from single-cell hPSCs are included in many commercially available kits and protocols and constitutes the starting point for the most of the PSC-staring neural differentiation protocols for the CNS populations.

Here the authors chose to add 2 differentiation protocols for dopaminergic neurons and astrocytes, as to show some potential applications.

Major Concerns:

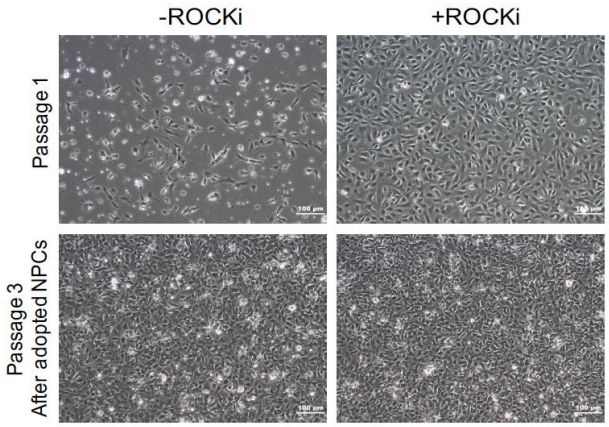
Back to the main goal of the paper and of Jove, what is the improvement of the neural induction that was shown in this paper and should be described in details?

If the improvement is the dissociation of the HESC colonies using accutase and Rock inhibitor, this was done starting with Watanabe 2007 (ref 7) and intensively used in the field. The presented results show similar growing of hESC in colony-type and single cell type cultures (Fig 1).

Response: As reviewer mentioned, we stated that various single cell passing methods have been published in which cells are cultured with ROCK inhibitor or small molecules to improve survival within 24 h, but their final products of these culture methods are colony-type hESCs. In contrast, in our high density single cell type culture, hESCs remain a single cell type cultures resulting in an improved and robust single cell culture system that is easily scalable. Through this culture system, hESCs can be easily maintained and expanded. In addition, we present an efficient method to generate NPCs from single cell type of hESCs that allows the production of highly pure NPCs. The data show that single cell culture of hESCs do not lose their ability to efficiently differentiate into NPCs. The subsequent differentiation into neurons and astrocytes was used to demonstrate the abilities of these single cell cultures, we were not trying to give the impression that these were new protocols. We revised the manuscript to avoid this impression.

If the improvement was to used the Rock inhibitors for neuroepithelial cells, this was also used before but not shown in all publication with neural induction. However, the authors are not showing here an increasing in surviving with and without the Rock inhibition.

Response: After 7 days of neural differentiation, we passed and cultured cells in NPC medium with/without ROCKi. As you can see, cells under -ROCKi condition at initial passage were shown lower survival rate compare to + ROCKi condition. However, after 3-4 passage with ROCKi condition, NPCs can be passed without ROCKi. Our protocol represents a combination of protocols previously used and reported. It makes expansion and maintenance of hESCs easier for larger scale experiments and high throughput screening.



Regarding the description of the methods, it is not clear what the main steps are. It is a need to be presented in text as in fig 4A.

Response: We focused on the single cell method for culturing hESCs and this protocol allows to a simple, robust, and reliable system for hESC culture. We then presented that these cells under single cell system have the potential to differentiate into neural cell type. Fig 4A showed differentiation schematic procedure through the adopted single cell culture of hESCs and we presented it in manuscript and figure legend. This is now clarified in section 4 of the Protocol.

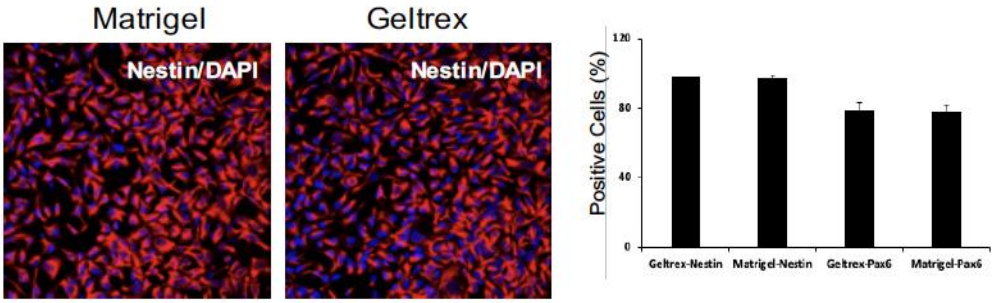
Proliferation sub-protocol should be presented separately. A sub-protocol for cryo-preservation is missing.

Response: As the reviewer's comments, we revised and corrected in revised manuscript.

It will be also of interest to say that there are variants for the matrix (Martigel, Geltrex, vitronectin, etc), for the PSC medium, for the neural induction medium (here is not clear if

DMEM, DMEM/F12 or Stemdiff neural progenitor medium is used). Of course many variants are here possible but they should be clarified and maybe compared.

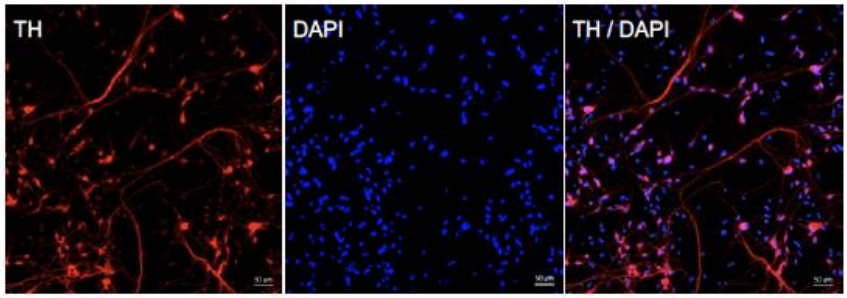
Response: As NPC culture medium, we are culturing NPCs in Stemdiff neural medium. To address the reviewer's suggestion, we made a comparison between Matrigel and Geltrex. Our data showed that NPC differentiation performed very similarly on both matrices as indicated by the similarities in expression of several NPC markers. This comparison was included in the revised version (Protocol 4 section).



Regarding the characterization of the neural cells after the neural induction (at day 7) it was shown in many publications, including in the previous one for the authors (Jeon 2019), that they represent neuroepithelial cells with embryonic CNS anterior phenotype. With their proliferation in FGF+EGF conditions, the profile becomes more posterior and is also related to the passage number, in parallel with a decrease in neuronal differentiation potential and increase in the glial potential. In these conditions, there is far to be well described and discussed the next steps of neuronal and glial differentiation (protocols 4 and 5). Additionally, the protocol 4 is not up-to-date and far to be efficient, comparing to the floor-plate related dopaminergic differentiation protocols. Here also the description of the results in fig 6 A is questionable, as the TH staining seems to be unspecific. I consider here as a critical step that the patterning and the neurogenesis potential to be discussed/compared and emphasized for different application types.

Response: We thank the reviewer for the suggestion. However, we didn't address any patterning related neural experiments in this paper. Here we focused on the single cell method for culturing hESCs and this protocol allows to a simple, robust, and reliable system for hESC culture. We then presented that these cells under single cell system can be showed a potential to differentiate into neural cell type and these cells under single cell type culture system can efficient and fast NPC differentiation within one week. In addition, we presented these NPCs have a potential to further differentiate into DA and Astrocyte. It was not our intend to focus on improving protocols for DA and astrocytes. We revised the paper to avoid giving this impression.

With regard to the TH staining: by mistake an image of Tuj-1/Map2 staining was used instead of TH. We exchanged the TH images (Sigma T1299, TH antibody). We apologize for this error.



Reviewer #2:

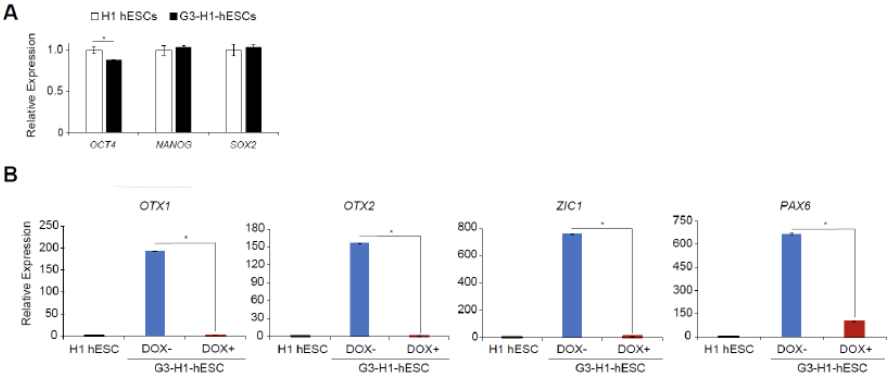
Manuscript Summary:

The manuscript "Efficient and Fast Neural Differentiation through Single Cell Type Culture of Human Embryonic Stem Cells" by Jeon et al establish a protocol for neural Differentiation from hESCs. These protocols are simple and efficient, and suitable for drug screening and regenerative medicine.

Minor Concerns:

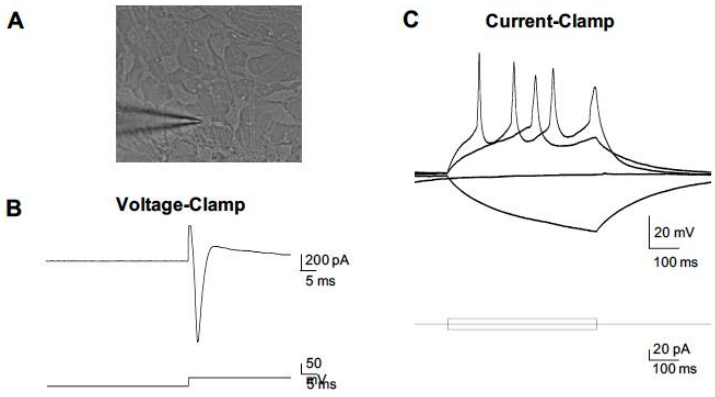
1. Only H9 is used in the study? Do you check the protocol in other cell lines, such as H1 and ipsc?

Response: We reported single cell culture of WA01 (H1) hESC in our previous published paper (Stem Cells. 2019 Feb;37(2):202-215. doi: 10.1002/stem.2941, with very similar results as WA09. We now stated this in the protocol.



2. How about the function of neural progenitor cells , in vivo or in vitro (not only staining)?

Response: We examined the function of DA neurons derived from NPCs and the representative recording action potentials in DA cells under the current clamp mode of a patch-clamp amplifier as below. We believe that this is outside the scope of this protocol and therefore was not included in the paper.



(A) DIC image of the recorded cell. (B) Voltage-clamp. (C) Current-clamp