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TITLE:

Differentiation and Characterization of Neural Progenitors and Neurons from Mouse Embryonic Stem Cells

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KEYWORDS:

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SUMMARY:

We describe the procedure for the in vitro differentiation of mouse embryonic stem cells into neuronal cells using the hanging drop method. Furthermore, we perform a comprehensive phenotypic analysis through RT-qPCR, immunofluorescence, RNA-seq, and flow cytometry.

ABSTRACT:

We describe the step-by-step procedure for culturing and differentiating mouse embryonic stem cells into neuronal lineages, followed by a series of assays to characterize the differentiated cells. The E14 mouse embryonic stem cells were used to form embryoid bodies through the hanging drop method, and then induced to differentiate into neural progenitor cells by retinoic acid, and finally differentiated into neurons. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) and immunofluorescence experiments revealed that the neural progenitors and neurons exhibit corresponding markers (nestin for neural progenitors and neurofilament for neurons) at day 8 and 12 post-differentiation, respectively. Flow cytometry experiments on an E14 line expressing a *Sox1* promoter-driven GFP reporter showed that about 60% of cells at day 8 are GFP positive, indicating the successful differentiation of neural progenitor cells at this stage. Finally, RNA-seq analysis was used to profile the global transcriptomic changes. These methods are useful for analyzing the involvement of specific genes and pathways in regulating the cell identity transition during neuronal differentiation.

INTRODUCTION:

Since their first derivation from the inner cell mass of the developing mouse blastocysts[1, 2], mouse embryonic stem cells (mESC) have been used as powerful tools to study stem cell self-renewal and differentiation[3]. Furthermore, studying mESC differentiation leads to tremendous understanding of molecular mechanisms that may improve efficiency and safety in stem cell-based therapy in treating diseases such as neurodegenerative disorders[4]. Compared to animal models, this in vitro system provides many advantages including simplicity in practice and assessment, low cost in maintaining cell lines in contrast to animals, and relative ease in genetic manipulations. However, the efficiency and quality of differentiated cell types are often affected by different lines of mESCs as well as the differentiation methods[5, 6]. Also, the traditional assays to evaluate differentiation efficiency rely on qualitative examination of selected marker genes which lack robustness and they therefore fail to grasp global changes in gene expression.

Here we aim to use a battery of assays for systematic assessment of the neuronal differentiation. Using both traditional in vitro analyses on selected markers and RNA-seq, we establish a platform for measurement of the differentiation efficiency as well as the transcriptomic changes during this process. Based on a previously established protocol[7], we generated embryoid bodies (EBs) through the hanging drop technique, followed by induction using supraphysiologic amount of retinoic acid (RA) to generate neural progenitor cells (NPCs), which were subsequently differentiated to neurons with neural induction medium. To examine the efficiency of the differentiation, in addition to traditional RT-qPCR and immunofluorescence (IF) assays, we performed RNA-seq and flow cytometry. These analyses provide comprehensive measurement of the progression of the stage-specific differentiation.

PROTOCOL:

1. mESC culture

1.1. Coat a 10 cm tissue-culture-treated plate with 0.1% gelatin and allow the gelatin to set for at least 15–30 min before aspirating it out.

1.2. Seed γ-irradiated mouse embryonic fibroblasts (MEFs) one day before culturing the mESCs in the pre-warmed mESC medium (Dulbecco's modified Eagle medium (DMEM) with 15% fetal bovine serum (FBS), non-essential amino acids, β-mercaptoethanol, L-glutamine, penicillin/streptomycin, sodium pyruvate, LIF, PD0325901 (PD), and Chir99021 (CH)).

1.3. Allow the γ-irradiated MEFs to settle and attach to the plate surface before culturing E14 cells.

1.4. Thaw E14 ESCs in 37 °C water bath and quickly transfer the cells in a 15 cm conical tube with warm mESC medium. Pellet the cells at 200 x *g* for 3 min and remove the supernatant.

1.5. Resuspend the cells in 10 mL of mESC medium and plate the cells on the culture plate containing the γ-irradiated MEFs seeded earlier. Incubate the cell culture in a 37 °C incubator under 5% CO_2.

1.6. For culture passaging, aspirate e the medium and wash the plate with sterile 1x PBS. Add enough 0.05% trypsin to cover the plate surface and incubate at 37 °C for 3 min.

1.7. Neutralize the trypsin with mESC medium and pipette to generate single-cell suspension. Centrifuge the cells at 200 x *g* for 3 min and remove the supernatant.

1.8. Count the cells with a hemocytometer or cell counter and seed about 5.0 x 10^5 cells in a 10 cm culture plate.

1.9. Resuspend the cells in 10 mL of mESC medium, plate the cells on the gelatin-coated tissue culture plate and incubate cultures as described earlier.

NOTE: It is recommended that mESCs be passaged every 2 days to prevent the cells from differentiating in their colonies. Phenol red in the medium functions solely as a pH indicator and depending on the cellular density, it can turn yellowish (more acidic), sooner than 2 days. Hence, it may be necessary to change the medium every day. The γ-irradiated MEFs will eventually die off after a couple of passages.

2. EB, NPC, and neuron differentiation

2.1. Perform culture passaging protocol mentioned earlier and count the cells (steps 1.7–1.10).

2.2. Hanging drop method (**day 0**)

2.2.1. For a 10 cm cell culture plate, count roughly 2.5 x 10^4 cells where 5.0 x 10^2 cells will be suspended in 20 µL differentiation medium (DMEM with 15% FBS, non-essential amino acids, β-mercaptoethanol, L-glutamine, penicillin/streptomycin, and sodium pyruvate). Roughly fifty 20 µL droplets containing the cells can be plated on one 10 cm plate.

2.2.2. Aliquot the appropriate number of cells, and then centrifuge the cells at 200 x *g* for 3 min and remove the supernatant.

2.2.3. Resuspend the cells in the appropriate volume of differentiation medium for a cell density of 5.0 x 10^2 cells per 20 µL (e.g., 2.5 x 10^4 cells in 1 mL of differentiation medium).

2.2.4. Using a micropipette or a repeater pipette, place 20 µL droplets of the cell suspension onto the lid of the tissue culture plate. Make sure that the droplets are not too close to one another to prevent them from merging.

NOTE: The droplets can be plated on either a tissue-culture-treated attachment plate or suspension plate as they will be placed on the lid and not the plate itself. For a more feasible and sterile approach, plate the droplets on an attachment tissue-culture plate and transfer them to a suspension plate as described below.

2.2.5. Fill up the plate with 5–10 mL of 1x PBS and carefully put the lid back on the plate. Incubate the culture in the 37 °C incubator.

NOTE: PBS is added to the culture plate to prevent the droplets from drying up.

2.3. On **day 2**, use a micropipette to collect the droplets from the lid and place them in a 10 cm cell culture suspension plate filled with 10 mL of differentiation medium. Incubate the culture on an orbital shaker shaking at low speed in the incubator.

2.4. On **day 4**, to harvest the EBs, collect the cells, centrifuge at 200 x *g* for 3 min, and remove the supernatant.

NOTE: The EBs can also be washed with 1x PBS as per the requirements of subsequent experiments.

2.5. To continue the procedure and induce the EB differentiation into neural progenitor cells (NPCs), prepare the differentiation medium with 5 µM retinoic acid (RA).

2.6. Remove the old medium by pelleting the EBs at 100 x *g* for 3 min or allow the EBs to settle before aspirating out the old medium. Add 10 mL of the differentiation medium containing 5 µM RA to the culture plate.

2.7. On **day 6**, replace at least half of the medium with fresh medium containing 5 µM RA by tilting the plate and pipetting out the medium as described above.

NOTE: It is recommended that at least half of the medium be replaced with fresh RA-containing medium on days 5 and 7. Take note of the phenol red indicator; if it turns yellowish, it is best to replace all of the medium.

2.8. On **day 8**, harvest the NPCs by collecting the cells, centrifuging at 200 x *g* for 3 min, and removing the supernatant.

NOTE: The NPCs can also be washed with 1x PBS according to the needs of subsequent experiments. If needed, NPCs can be frozen down and thawed again for later culture and analysis. If the NPCs are to be cultured, accutase can also be used as an alternative to trypsin.

2.9. To continue the procedure and to differentiate NPCs into neurons, collect NPCs in a 15 mL conical tube by centrifugation, dissociate them with trypsin and incubate them at 37 °C for 3 min. Pipette the NPCs to ensure that all NPC aggregates are dissociated and neutralize the trypsin with the medium.

2.10. Filter the cells with 40 µm nylon cell strainer and count the cells before plating them at a density of 1.5 x 10^5/cm^2 in N2 medium (DMEM/F12 medium + 3 mg/mL glucose + 3 mg/mL lipid-

rich bovine serum albumin (LBSA) + 1:100 N2 supplement + 10 ng/mL bFGF + 50 U/mL pen/strep + 1 mM L-glutamine) on a tissue-culture-treated plate for subsequent PCR and western blot experiments; or on a tissue culture chamber for immunofluorescence experiments.

2.11. On **day 9**, replace the old medium with a fresh N2 medium.

2.12. On **day 10**, switch the N2 medium with N2/B27 medium (50% DMEM/F12 and 50% neural basal, 3 mg/mL LBA, 1:200 N2 supplement, 1:100 B27 supplement, 50 U/mL pen/strep, and 1 mM L-glutamine).

2.13. On **days 11–12**, harvest the neurons as follows. Wash the cells with 1x PBS, add trypsin, and incubate the culture in the 37 °C incubator for 3 min before neutralizing the trypsin with medium and centrifuge at 200 x *g* for 3 min.

3. Characterization of mESCs and differentiated cells

3.1. Alkaline phosphatase (AP) assay

3.1.1. Use a kit to assess alkaline phosphatase activity (see the **Table of Materials**).

3.1.2. Remove the medium from the culture plate and wash the ESCs with 1x PBS.

3.1.3. Add 1 mL of the fix solution (consists of formaldehyde and methanol) provided with the kit to the plate and incubate it at room temperature for 2–5 min.

NOTE: Over-incubation in the fix solution can compromise the AP activity.

3.1.4. Remove the fix solution, wash the ESCs with 1x PBS and leave some amount of PBS in the plate.

NOTE: Keep the ESCs moist in PBS to not compromise the AP activity.

3.1.5. Prepare the AP solution by mixing the A, B, and C substrate solutions at 1:1:1 ratio. Mix A and B solutions first and incubate the mixture at room temperature for 2 min before adding the C solution.

3.1.6. Remove the 1x PBS and add the AP solution prepared earlier.

3.1.7. Incubate the ESCs for about 15 min in the dark by wrapping the culture plate with aluminum foil or performing step 3.5 in a dark room.

3.1.8. Monitor the reaction and remove the reaction solution when the solution turns bright to avoid non-specific staining.

3.1.9. Wash the ESCs twice with 1x PBS.

3.1.10. Prevent the sample from drying by covering the ESCs with 1x PBS or mounting medium.

NOTE: A red or purple stain will appear for AP expression. The plate can be stored in a 4 °C refrigerator.

3.2. **RT-qPCR**

3.2.1. Collect cells at various stages by following steps 1.6–1.7 and 2.4 for ESCs and EBs and NPCs, respectively.

3.2.2. Isolate RNA using RNA, DNA, and protein extraction solution (see **Table of Materials**).

3.2.3. Generate cDNA with a reverse transcriptase kit (see the **Table of Materials**) and follow the manufacturer's manual.

3.3. **Fixation and embedding**

3.3.1. Harvest EBs and NPCs as described above (step 2.6) and fix them with 4% paraformaldehyde (PFA) solution in 1x PBS for 30 min at room temperature.

3.3.2. Remove the PFA and wash the sample with 1x PBS for 5 min.

3.3.3. Place the sample in a serial dilution of 1x PBS, 10%-, 20%-, and 30%-sucrose solutions at 25–28 °C where the sample is transferred to the next solution after 30 min of incubation.

NOTE: The sample can be stored in the 30% sucrose solution at 4 °C before continuing with the embedding step.

3.3.4. Wet the pipette tip with sucrose solution before placing the sample (without stacking them) at the center of the cryo-mold and pipette out the excess liquid.

NOTE: Filter paper can also be used to remove the excess solution. Wetting the pipette tip with sucrose solution is important to prevent the EBs and NPCs from sticking to the walls of the tip.

3.3.5. Carefully add optimum cutting temperature (OCT) solution to the mold without resuspending the samples and remove excess bubbles with a pipette.

3.3.6. Place the mold with the sample on a laboratory mixer at low speed to mildly agitate the sample for 15 min. This helps to settle the EBs and NPCs to the bottom if they are resuspended in the OCT solution.

3.3.7. Quickly freeze the sample by placing the mold in liquid nitrogen or on dry ice.

NOTE: Samples can be stored in a -70 °C freezer before continuing to the next step.

3.4. **Cryosectioning**

3.4.1. Set the cryostat to cool down to -20 to -18 °C before transferring the sample to the instrument.

3.4.2. Detach the frozen OCT block from the mold and secure it on the holder with a little OCT solution placed at the surface of the holder.

3.4.3. Align the OCT block so that the EBs and NPCs are closest to the blade to ensure that the sample is not lost during sectioning.

3.4.4. Carefully section 10 µm of the block and pay close attention to the slices that contain the sample.

3.4.5. Quickly place the OCT slice containing the sample onto the tissue-embedding glass slide and allow the OCT slices to air-dry for 1 h at room temperature.

NOTE: The samples can be stored at -70 °C for later use.

3.5. **Immunofluorescence (IF)**

3.5.1. Block OCT sections or culture chambers containing neurons in 10% normal donkey serum/0.1% Triton X-100 in 1x PBS for 1 h at room temperature.

3.5.2. Incubate the samples in primary antibody diluted in 5% normal donkey serum/0.05% Triton X-100 in 1x PBS overnight at 4 °C.

3.5.3. Wash samples in 1x PBS/0.1% Triton X-100 thrice for 5 min each wash.

3.5.4. Incubate the samples with secondary antibody diluted in 5% normal donkey serum/0.05% Triton X-100 in 1x PBS for 1 h at room temperature.

3.5.5. Wash samples in 1x PBS/0.1% Triton X-100 thrice for 5 min each wash the incubate the samples in 1 µg/mL DAPI.

3.5.6. Mount the samples with a coverslip and some mounting medium and allow it to dry.

3.5.7. Observe the samples under a fluorescence microscope.

3.6. **RNA-seq analysis**

3.6.1. Collect the cells at various stages and perform RNA extraction (see step 3.2).

3.6.2. Prepare the cDNA libraries, perform deep sequencing and data analysis according to the protocol described in Wang et al.[8].

3.6.3. Perform the Gene Ontology (GO) analysis using the R package, clusterProfiler.

3.7. **Flow cytometry**

3.7.1. Collect ESCs by following the steps 1.6–1.7 and resuspend the cells in medium. Collect the EBs and NPCs by following step 2.4 and resuspend the cells in medium.

3.7.2. Filter the cell suspension using the 40 µm nylon cell strainer into a new 15 mL conical tube.

3.7.3. Measure the GFP signal of the samples using the flow cytometer (performed by the institution's core facility).

REPRESENTATIVE RESULTS:

As a representation of our method, we performed an EB, NPC, and neuron differentiation experiment on E14 cells. E14 cells were cultured on γ-irradiated MEFs (**Figure 1A**) until the γ-irradiated MEF population diluted out. We confirmed the pluripotency of the E14 cells by performing Alkaline Phosphatase (AP) staining (**Figure 1B**) and later RT-qPCR (see below) for *Nanog* and *Oct4* markers. The γ-irradiated MEF-free E14 cells were then induced for differentiation using the protocol outlined in **Figure 2A**. Briefly, differentiation media droplets of 20 µL containing 500 cells were seeded on the lid of the culture plate (see protocol section 2 for details). The EBs formed were then collected and placed in suspension in fresh differentiation media. From day 4 to day 8 of differentiation, 5 µM RA was added to the culture plates to induce NPCs. Differentiated EBs showed round shape and their size continued to increase during differentiation (**Figure 2B**). At day 8, the NPCs were harvested and trypsinized, and then the resulting single cell suspension was plated in a tissue culture chamber in DMEM/F12 medium with N2 supplement and later in B27 supplement. By day 10, NPCs differentiating into neurons appear to have elongated-cell shape (**Figure 2B**).

To further evaluate our differentiation experiment, we performed immunofluorescence (IF) experiments on E14 NPCs at day 8 and E14 neurons at day 12. We observed positive staining for nestin in NPCs and neurofilament (NF) signal for neurons (**Figure 3A**). Alternatively, RT-qPCR and RNA-seq confirmed the induction of NPC marker genes and loss of pluripotency genes in NPCs (**Figure 3B,D,F**). As a quantitative method to test the success of ESC differentiation, we differentiated a mouse ESC line expressing a *Sox1* promoter-driven GFP reporter[9], followed by flow cytometry analysis on ESCs and NPCs. We found that 58.7% of total cells at the NPC stage are GFP-positive while the GFP signal is 0.0% at the ESC stage (**Figure 3C**). To profile the transcriptomic changes during differentiation, RNA-seq experiments for E14 ESCs, EB day 3, and NPC day 8 were performed and revealed gene clusters associated with the respective stages (**Figure 3D**). The genes in the RNA-seq heat-map were sorted based on their expression levels to

identify differentially expressed genes in the different stages during differentiation. Gene Ontology (GO) analysis for the four gene clusters showed that these clusters correspond to distinct cellular functions or pathways indicating that the three cell stages of mESC neuronal differentiation each have a group of genes that are highly expressed in their respective stage but not others (**Figure 3E**). For example, genes in Cluster 3 are highly expressed in E14 NPCs compared to other stages and correspond to pathways related to neuronal development. Clusters 1, 2, and 4 do not contain highly expressed genes related to any germ layer lineage specifications but they are related to cellular growth and proliferation. Thus, the RNA-seq and accompanying GO analysis showed that the E14 cells have differentiated into the neuronal lineage by day 8 of differentiation.

FIGURE AND TABLE LEGENDS:

Figure 1: E14 ESCs in culture. (**A**) The light microscope images show E14 cells (black arrow) growing in colonies atop the γ-irradiated MEFs. E14 colonies continue to proliferate as seen in the colony size difference between day 1 and day 3 cultures. (**B**) Confirmation of the pluripotency of E14 ESCs by the alkaline phosphatase (AP) stain. Purple arrows indicate the mESCs that were positive for AP stain.

Figure 2: E14 differentiation into EBs, NPCs, and neurons. (**A**) The schematic summarizes the major steps for differentiating E14 cells into EBs, NPCs, and neurons. (**B**) E14 ESCs cultured in medium without LIF and 2i in a suspension plate form individual spheres of EBs visible at day 2 where they continue to grow and expand in size in subsequent days. RA is added at day 4 of differentiation to induce the differentiation into NPCs. After 4 days of induction, these NPCs are plated for differentiation into neurons, which are shown in the bottom panel.

Figure 3: Characterization of differentiated cells. (**A**) Immunofluorescence images in the top panel show NPC-containing EBs at day 8 probed for nestin (green) and nuclei (DAPI, blue). The bottom panel shows immunofluorescence images for neurons at day 12 probed for neurofilament (green). The red box in the merged images are zoomed in 3x for better view. (**B**) RT-qPCR analysis showing the pluripotency markers *(Nanog* and *Oct4)* and NPC markers *(Pax6*, *NeuroD1*, and *Nes)* of E14 ESCs and NPCs. Error bars are mean ± SD. (**C**) E14 cells expressing a *Sox1* promoter-driven GFP reporter were differentiated into NPCs. Both the ESCs and NPCs at day 8 were quantified for positive GFP fluorescent signal with flow cytometry. (**D**) The heatmap shows the z-scores for the determined FPKM of the genes expressed in the ESC, EB, and NPC stages. Four distinct gene clusters were identified signifying groups of genes that are differentially expressed in either the ESC, EB, or NPC stage. (**E**) GO analysis was performed using the R package, clusterProfiler, on the four clusters identified in the RNA-seq. (**F**) The graph shows the FPKM values for three other pluripotency markers, *Sox2*, *Klf4*, and *Myc* as well as neural markers, *NeuN*, *Map2*, and *Tubb3* for E14 cells at the ESC, EB day 3, and NPC day 8 stages.

DISCUSSION:

The method for neural differentiation of mouse embryonic stem cells has been established for decades and researchers have continued to modify the previous protocols or create new ones

for various purposes[7, 10, 11]. We utilized a series of assays to comprehensively analyze the efficiency and progress of the differentiation stages of mESCs to neurons, which may be used in analysis of other lineage differentiation of mouse or human ESCs. Furthermore, our approaches have proved to be useful tools to evaluate the impact of specific genes or pathways on neuronal differentiation in vitro[8].

With our methods, pluripotent un-committed ESCs treated with retinoic acid (RA) commit to the neural lineage with a high efficiency and are further induced to generate neurons[7]. To improve the successful differentiation of ES cells into neural cells and reduce the heterogeneity, it is important to keep the ES cells in an undifferentiated state[12]. Non-proliferative MEFs, treated with γ-irradiation or mitomycin C, function to maintain the pluripotency of the ESs and provide a scaffold for their growth[13, 14]. To obtain consistent results, we start each differentiation experiment with mESCs cultured on γ-irradiated MEFs. After a few passages, the γ-irradiated MEFs die out and the culture eventually becomes homogenous for mESC cells. Alternatively, the mESCs can be pre-plated on gelatin for about 45 min before γ-irradiated MEFs are seeded to better remove them on the next passage. Leukemia inhibitory factor (LIF) has long been used to maintain the pluripotency state of cultured mouse ES cells by activating the JAK/STAT pathway[15–17]. More recently, PD0325901 (PD, a MEK inhibitor) and CHIR99021 (CH, a GSK inhibitor) were found to provide additional pluripotency maintenance of the ES cells[3, 18]. In our protocol, we culture mESCs with these inhibitors together with LIF to maintain high pluripotency of mESCs.

Another critical factor to achieve successful differentiation is the quality of EBs. We perform the differentiation of E14 cells by the hanging drop method, which has been applied by other investigators[5, 19, 20]. With this method, single ES cells are allowed to suspend in the differentiation medium droplet for 2 days where they spontaneously aggregate and form EBs. The resulting EBs are typically more well-defined in terms of their morphology (**Figure 2B**) compared to the method of suspending isolated mESCs in medium, which results in EB sizes in a much wider range in our experience (data not shown). To prevent EBs from attaching to the plates, it is important to do a low speed rotation starting from day 3 continuing through the differentiation process. The EBs are induced to differentiate into NPCs by treating them with RA. The resulting NPCs from RA treatment at EB day 4 are typically heterogenous for neural cells such as oligodendrocytes and astrocytes[21]. Using RT-qPCR or immunofluorescence, the NPC population can be probed for neuron and other neural lineage markers such as *Gfap* for astrocytes and *Olig2* for oligodendrocytes. To further induce the differentiation of NPCs into neurons, the NPCs are cultured in optimal neuron medium where the most important components are the N2 and B27 supplements. N2 supplement mainly functions to help the NPCs to commit to the neuronal lineage while the B27 supplement functions to maintain the longevity of the neurons.

The samples can be collected across the differentiation period (e.g., ESC stage, EB day 2, EB day 4, NPC day 6, NPC day 8, neurons day 10, and neurons day 12) to track the differentiation process by performing RT-qPCR for pluripotency and ectoderm markers. Comparing the pluripotency markers such as *Oct4*, *Nanog*, *Sox2*, *Klf4*, and *Myc* between the different cell stages will verify the pluripotency of the mESCs (**Figure 3B,F**). To investigate the efficiency of the neural differentiation, markers for the mesoderm layer such as *Hand1*, *Snai1*, and *Tbxt*; and endoderm

layer such as *Eomes* and *Gata4* can also be probed for (data not shown). Further verification can be performed with immunofluorescence (IF) probing for NPC or neuronal markers (**Figure 3A**). However, these methods are not quantitative and biased toward the selected markers. To overcome these limitations, we incorporate flow cytometry and RNA-seq analyses (**Figure 3C–F**). The cell line used in the flow cytometry experiment is a *Sox1-GFP* E14 cell line, which was used specifically in this experiment to assess the quality of the NPC differentiation procedure. *Sox1* is one of the earliest specific neuronal marker during neuroectoderm development[22] hence making it an excellent marker for NPC lineage. *Sox1* can be probed for using RT-qPCR or Western blot to evaluate the NPC population. These analyses are particularly beneficial to investigate the differentiation defect caused by gene manipulation or chemical treatment.

It is important to note that there are a few limitations to our protocol presented here. First of all, we are only presenting the comprehensive analysis for one wild-type mESC cell line. Other ESC lines originating from mice or humans might require changes and further optimization in the protocol to ensure successful and efficient neuron differentiation. Secondly, we present an in vitro neuron differentiation method, which naturally bears its own set of limitations. As mentioned before, EBs are treated with a supraphysiological level of RA to drive them towards the NPC lineage. The resulting NPCs are then placed in neuron-optimum media to mimic the physiological conditions and encourage neuron lineage commitment, growth, and longevity. Here, N2 and B27 supplements are used to culture neurons but other supplements are also available such as NS21[23] for similar purposes, which may alter the success and efficiency of neuron differentiation. These conditions are synthetically reconstituted in the cell culture assays, which may not fully represent physiological conditions. The quality of the EBs, NPCs, and neurons highly depend on the starting mESCs. mESCs that have been passaged for too many times and kept in culture for more than 1 week typically start to lose pluripotency and may not successfully undergo differentiation. Thus, maintaining the mESCs in an optimal condition is key in ensuring that they can effectively differentiate into EBs, NPCs, and neurons. Other neuron culture methods such as 3D models have also been proposed to better mimic physiological conditions[24–26] sometimes at the expense of throughput and feasibility[27, 28]. We believe our protocols are useful to characterize these 3D culture models.

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DISCLOSURES:

Authors declare that there are no competing financial interests.

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Figure

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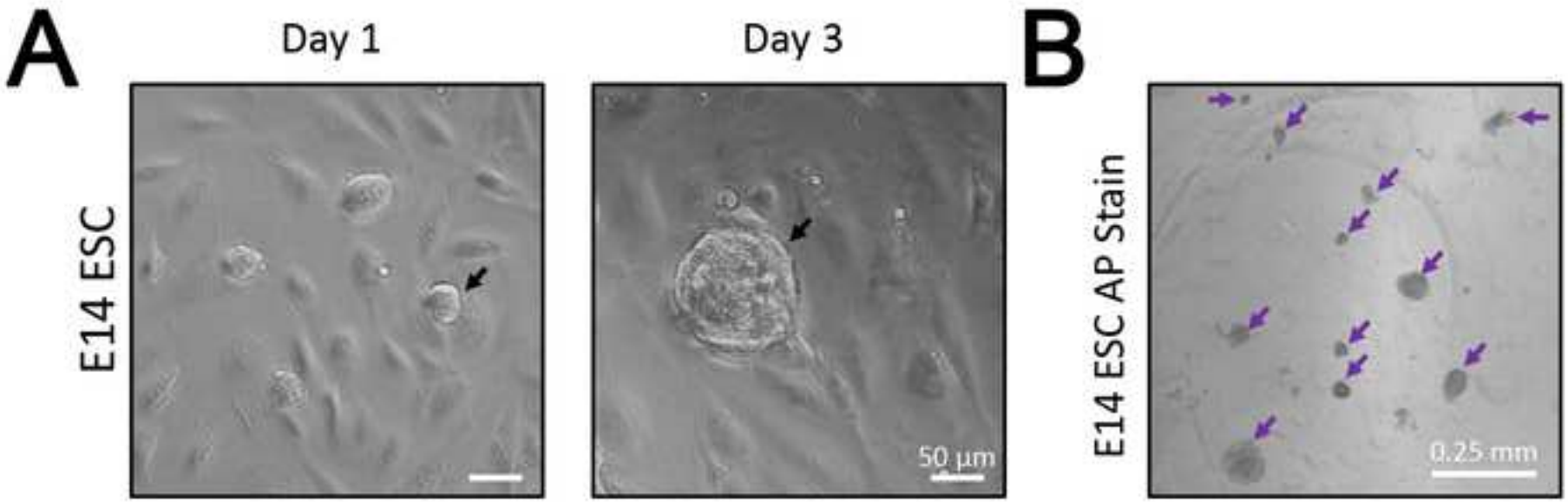
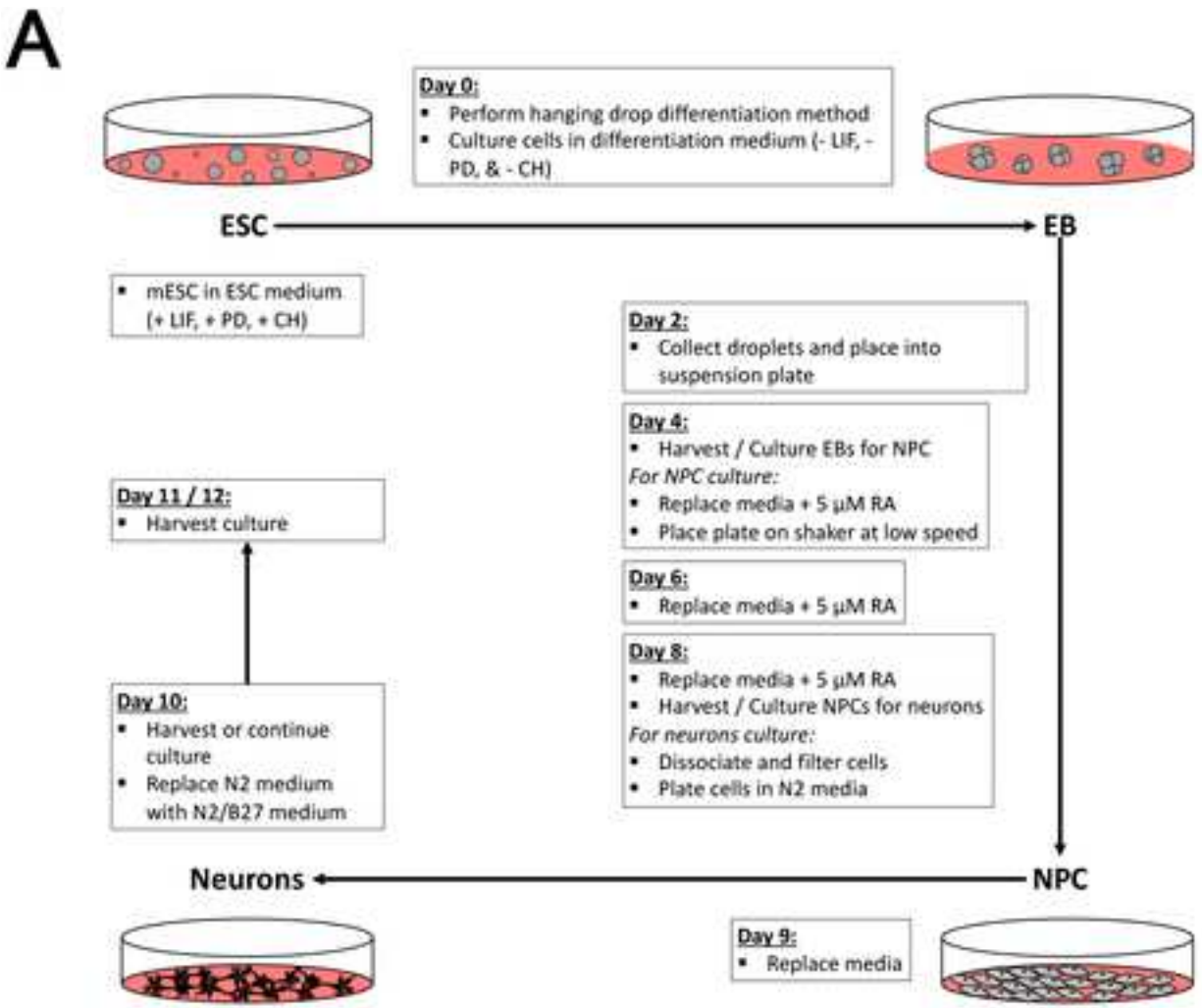
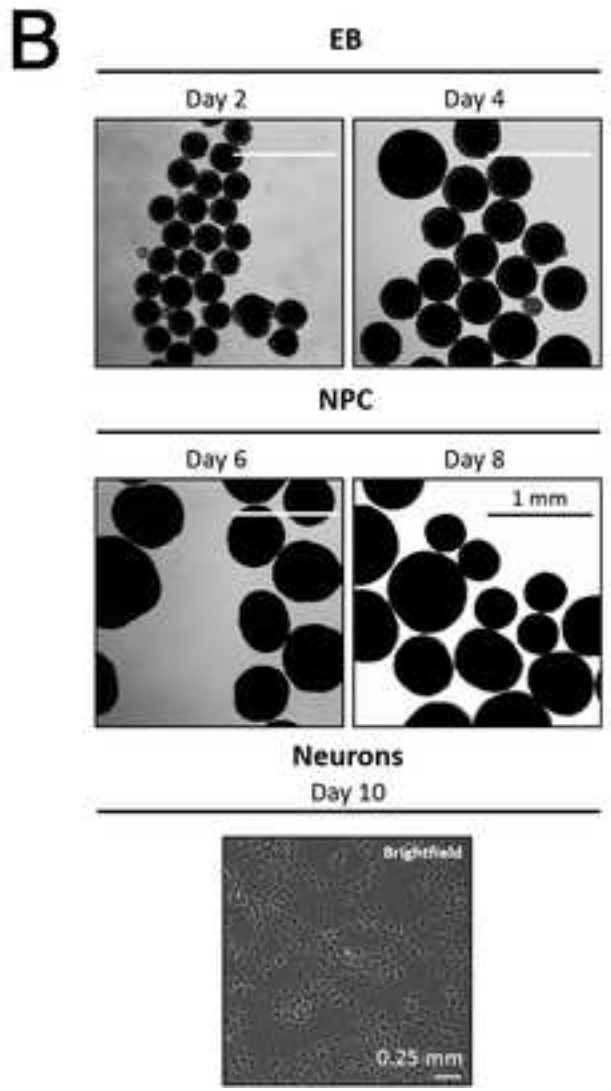


Figure





Figure

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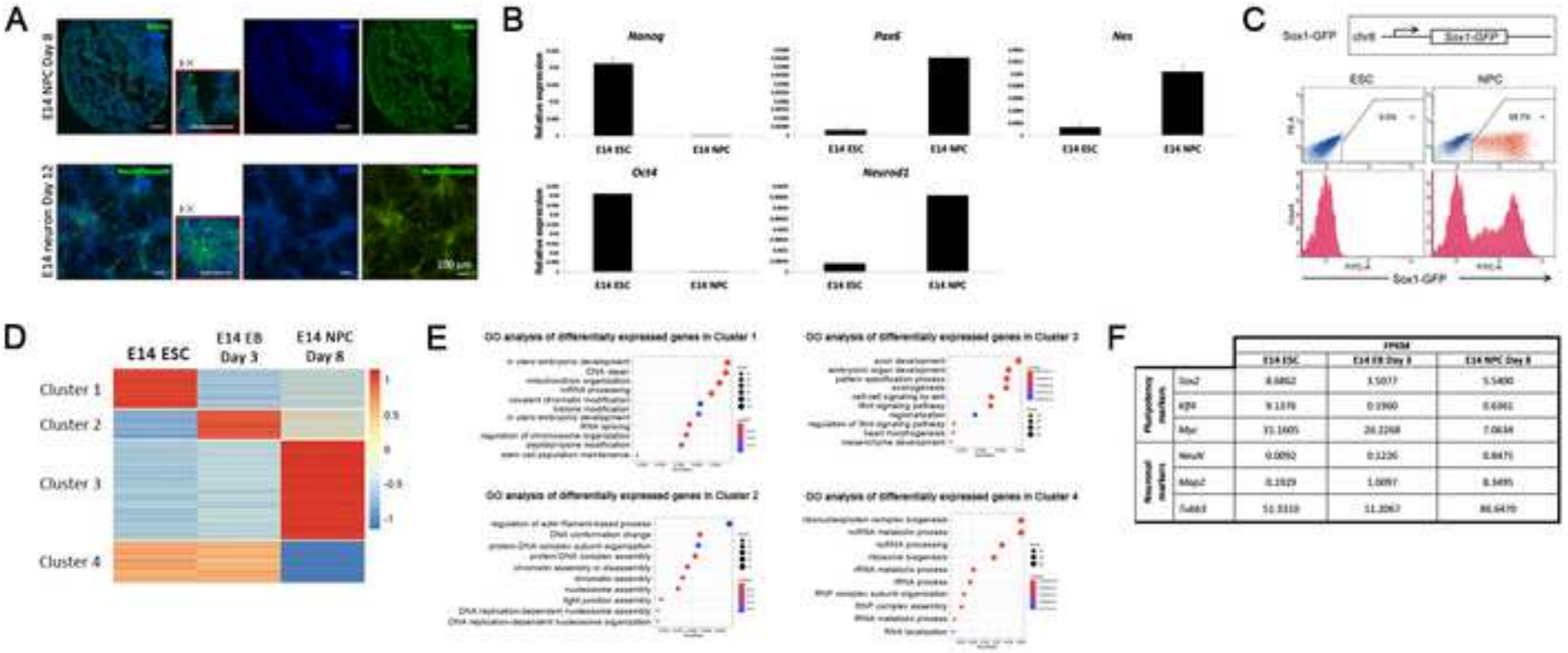

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.05% Trypsin + 0.53mM EDTA 1X	Corning	25-052-CV	
0.1% Gelatin	Sigma	G1890-100G	Prepared in de-ionized water
16% Paraformaldehyde	Thermo Scientific	28908	Diluted in 1X PBS
40-µm cell strainer	Falcon	352340	
Albumax	Thermo Fisher Scientific	11020021	
AlexaFluor 488 goat anti-mouse IgG (H+L)	Invitrogen	A11001	Antibody was diluted at 1:500 for IF
Alkaline Phosphatase Staining Kit II	Stemgent	00-0055	
AzuraQuant Green Fast qPCR Mix LoRox	Azura Genomics	AZ-2105	
B27 supplement	Thermo Fisher Scientific	17504044	
BD FACSCanto	BD	657338	
bFGF	Sigma	11123149001	
BioAnalyzer High Sensitivity DNA Kit	Agilent	5067-4626	
Chir99021	Cayman Chemicals	13122	
Chloroform	C298-500	Fisher Chemical	
DAPI	Invitrogen	R37606	
DMEM	Corning	10-017-CM	
DMEM/F12 medium	Thermo Fisher Scientific	11320033	
EB buffer	Qiagen	19086	
Ethanol	111000200	Pharmco	Diluted in de-ionized water
Fetal bovine serum	Atlanta Biologicals	S10250	
Fisherbrand Superfrost Plus Microscope Slides	Fisher Scientific	12-550-15	
HiSeq 2500 Sequencing System	Illumina	SY-401-2501	
Isopropanol	BDH1133-4LG	BDH VWR Analytical	Diluted in de-ionized water
L-glutamine	Thermo Fisher Scientific	25030024	
LIF	N/A	N/A	Collected from MEF supernatant
m18srRNA primers	IDTDNA	N/A	5'-GCAATTATTCCCCATGAACG-3' 5'-GGCCTCACTAAACCATCCAA-3'
MEM Non-essential amino acids	Corning	25-025-Cl	
mNanog primers	IDTDNA	N/A	5'-AGGCTTTGGAGACAGTGAGGTG-3' 5'-TGGGTAAGGGTGTTCAAGCACT-3'
mNes primers	IDTDNA	N/A	5'-AGTGCCCAGTTCTAGTGGTGTCC-3' 5'-CCTCTAAAATAGAGTGGTGAGGGTTG-3'
mNeuroD1 primers	IDTDNA	N/A	5'-CGAGTCATGAGTGCCCAGCTTA-3' 5'-CCGGGAATAGTGAAACTGACGTG-3'
mOct4 primers	IDTDNA	N/A	5'-AGATCACTCACATCGCCAATCA-3' 5'-CGCCGGTTACAGAACCATACTC-3'
mPax6 primers	IDTDNA	N/A	5'-CTTGGGAAATCCGAGACAGA-3' 5'-CTAGCCAGGTTGCGAAGAAC-3'
N2 supplement	Thermo Fisher Scientific	17502048	
Nestin primary antibody	Millipore	MAB5326	Antibody was diluted at 1:200 for IF
Neural basal	Thermo Fisher Scientific	21103049	
Neurofilament primary antibody	DSHB	2H3	
NEXTflex Illumina Rapid Directional RNA-Seq Library Prep Kit	BioO Scientific	NOVA-5138-07	
PD0325901	Cayman Chemicals	13034	
Penicillin/streptomycin	Corning	30-002-Cl	
Phosphate-buffered saline (PBS)	N/A	N/A	Prepared in de-ionized water
- Potassium chloride	P217-500G	VWR	
- Potassium phosphate monobasic anhydrous	0781-500G	VWR	
- Sodium chloride	BP358-10	Fisher Bioreagents	
- Sodium phosphate, dibasic, heptahydrate	SX0715-1	Milipore	
Random hexamer primer	Thermo Scientific	SO142	
Retinoic acid	Sigma	R2625	Prepared in DMSO
Sodium pyruvate	Corning	25-000-Cl	
Sucrose	Sigma	84097	Diluted in 1X PBS
SuperScript III Reverse Transcriptase	Invitrogen	18064022	
Tissue-Tek O.C.T. compound	Sakura	4583	
TriPure Isolation Reagent	Sigma-Aldrich	11667165001	
TruSeq Rapid	Illumina	20020616	
β-mercaptoethanol	Fisher BioReagents	BP176-100	

Response to Reviewers for Manuscript Entitled "Differentiation and characterization of neural progenitors and neurons from mouse embryonic stem cells"

Dear editor and reviewers,

First and foremost, we would like to thank the three reviewers who have read and reviewed our manuscript for their time and effort. Based on the comments received, we have made changes where necessary and provided further explanations and justifications for other parts. We hope that our revised manuscript aptly eliminates the concerns raised by the reviewers and that it is ready for publication. Below we provide the point-by-point response to address the comments by the reviewers.

Reviewer #1

Manuscript Summary:

This protocol paper by Hanafiah and colleagues describes the step-by-step procedure for culturing E14 mESCs into neuronal lineages using embryoid bodies through the hanging drop method, and differentiating them using retinoic acid, followed by a series of assays to characterize the differentiated cells via RT-qPCR, immunofluorescence, flow cytometry, and RNA-seq analysis.

Major Concerns:

Abstract:

1. "Immunofluorescence experiments revealed that the neural progenitors and neurons exhibit corresponding markers…" please specify those markers.

   Response: Neural progenitor and neuron markers mentioned.

Introduction:

2. Authors mentioned: "Compared to animal models, this *in vitro* system provides many advantages including simplicity in practice and assessment, low cost in maintaining cell lines in contrast to animals, and relative ease in genetic manipulations." Although right, *in vitro* systems might not recapitulate the normal physiological environment of the body. Kindly, elaborate more on this point and focus on the importance of 3-dimensional (3D) culturing in this context and cite more papers such as:

   *Bahmad, H. F., Cheaito, K., Chalhoub, R. M., Hadadeh, O., Monzer, A., Ballout, F., ... & Abou-Kheir, W. (2018). Sphere-formation assay: three-dimensional in vitro culturing of prostate cancer stem/progenitor sphere-forming cells. Frontiers in oncology, 8, 347.
   *Bahmad, H. F., Mouhieddine, T. H., Chalhoub, R. M., Assi, S., Araji, T., Chamaa, F., ... & Abou-Kheir, W. (2018). The Akt/mTOR pathway in cancer stem/progenitor cells is a potential therapeutic target for glioblastoma and neuroblastoma. Oncotarget, 9(71),

33549.
*Bahmad, H. F., Chamaa, F. O., Assi, S., Chalhoub, R. M., Abou-Antoun, T., & Abou-Kheir, W. (2019). Cancer stem cells in neuroblastoma: expanding the therapeutic frontier. Frontiers in molecular neuroscience, 12, 131.
*Bodgi, L., Bahmad, H. F., Araji, T., Al Choboq, J., Bou-Gharios, J., Cheaito, K., ... & Abou-Kheir, W. (2019). Assessing Radiosensitivity of Bladder Cancer In Vitro: A 2D vs 3D Approach. Frontiers in oncology, 9, 153.

Response: We agree with the reviewer that *In vitro* assays are limited in that they cannot physiologically recapitulate the biological mechanics of an organism to the extent that *in vivo* assays can. It is a great point to raise and we have briefly discussed this matter as a disadvantage of our *in vitro* assay. We appreciate the reviewer for providing us a few references to look into.

Protocol:

3. Specify expected timing for the MEFs to attach: "Allow for MEFs to settle and attach to the plate surface before culturing E14 cells."

   Response: We mentioned that MEFs can be plated a day before culturing the E14 cells but it might be better to mention this in the step itself. Thank you.

4. Give more details on the mESC medium and differentiation medium.

   Response: The contents of the mESC and differentiation media are mentioned in steps 1.2 and 2.2, respectively and are listed in our list of materials. We briefly mentioned that LIF, PD, and CH are used in the mESC medium to maintain the mESCs in their pluripotency state in the discussion section. We now have also mentioned that the absence of these inhibitors in the differentiation medium will allow the mESCs to differentiate into progenitors of the three germ layers.

5. "Fill up the plate with 5 - 10 mL PBS and carefully put the lid back on the plate." in section 2.6. Add why PBS is added.

   Response: We have now mentioned that PBS is added to keep the culture environment moist hence preventing the droplets from drying up.

6. Abbreviations (such as NPC) should be defined on their first use.

   Response: We have defined NPC again in the protocol section for clarification.

7. section 2.14: "If the phenol red indicator in the medium turns yellowish…" does phenol red affects cultured cells? Mention that.

Response: Thank you for pointing this out. We have now explained that phenol red functions as a pH indicator in the media and it does not necessarily affect our mESC culture itself. Although there are reports that estrogen-sensitive cells such as mammary cells and bone marrow stromal cells can respond to phenol red as it can it mimic the actions of estrogen.

8. When writing cell numbers, authors need to be consistent. Either write the whole number (50,000) or 5 × 104.

   Response: Thank you for this comment. We have changed all numbers mentioned in the manuscript to exponent form where relevant.

9. Section 2.19: "Switch the N2 medium with N2/B27 medium." Authors should mention the importance of adding B27 and making this switch.

   Response: This is an excellent point. We have now mentioned in the discussion section that B27 supplement is added in our assay to support the culture of neurons arising from embryonic stem cells.

10. All through the protocol, authors should specify timings. For example, in 2.20, "wash the cells with PBS, add trypsin …" For how many minutes trypsin should be added?

    Response: Thank you for picking on this. We have now mentioned throughout our protocol that cells are washed with PBS for 1 minute and left in trypsin for 3 minutes.

11. Alkaline phosphatase (AP) assay: Use the Alkaline Phosphatase Staining Kit II (Stemgent, Cat #00-005).
12. Section 3.3: "Add 1 mL of the Fix solution provided with the kit…" please specify the characteristics of the Fix solution.

    Response: Thank you for this comment. The Fix solution comes from the kit we used and we have mentioned that the it contains formaldehyde and methanol.

13. Add scale bars to all figures.

    Response: Thank you for pointing this out. We have added the scale bars to all cell images.

Results:

14. To confirm pluripotency, Nanog and Oct4 markers were used. How about other pluripotency markers such as Sox2, Klf4, and Myc. For neural differentiation, NeuN marker should have been used to confirm it.

Response: Thank you for mentioning this. We have checked the Sox2, Klf4, and Myc levels in our RNA-seq data and it shows that the levels for all three markers decrease in the EB and NPC stages compared to the mESC stage. We also checked for the transcript level of NeuN along with other neuronal markers such as Tubb3 and Map2 and we observed an increase in their transcript level from the mESC stage to the neuron stage. These analyses have been added into Fig 3.

15. For the RNA-seq experiment, authors only mentioned "revealed gene clusters associated with the respective stages." More results should be mentioned. Also, with respect to gene ontology (GO), authors said "analysis for the four gene clusters showed that these clusters correspond to distinct cellular functions or pathways." What is the relevance of those results in correspondence to the protocol presented?

    Response: We appreciate your concern on this matter. To further elaborate, we included in the results for the RNA-seq heat-map that the genes were sorted based on their expression level to identify differentially expressed genes in the different stages of the cell. The resulting four gene clusters indicate that the three cell stages of mESC neuronal differentiation each have a group of genes that are highly expressed in their respective stage but not others. For example, genes in Cluster 3 are highly expressed in E14 NPCs compared to other stages correspond to pathways related to neuronal development. Clusters 1, 2, and 4 do not contain highly expressed genes related to any germ layer lineage specifications but they are related to cellular growth and proliferation. Thus, the RNA-seq and accompanying GO analysis showed that the E14 cells have differentiated into the neuronal lineage by day 8 of differentiation.

Discussion:

16. The protocol presented herein does not represent novelty. They mentioned that they "utilized a series of assays to analyze the differentiation stage and efficiency, which may be used in analysis of other lineage differentiation of mouse or human ESCs." In fact, authors just compiled assays together and this should be mentioned.

    Response: We totally agree with the reviewer. Our goal is to present a comprehensive analysis that can be applied to evaluate the neuronal differentiation process.

17. The protocol has number of limitations, especially that only one cell line was used. Please add a section and mention all study limitations in it. Also, the importance of the protocol in regards to its application should be mentioned in the conclusion.

    Response: We appreciate you pointing this out. There are indeed limitations to our protocol. In our discussion, we are mentioning other limitations such as only one cell line was used in our protocol. Other limitations that we have identified pertaining to this protocol include the successfulness of the NPC differentiation and longevity of differentiated neurons. Although briefly mentioned, we are elaborating on the fact that checking for mesoderm (*Hand1*, *Snai1*, *Tbxt*) and endoderm (*Eomes*, *Gata4*) markers

with RT-qPCR in the different cell stages will inform us how successful the NPC differentiation is. Neurons cultured from differentiated stem cells do not have a long lifespan. The B27 supplement functions to maintain the neurons in an optimal condition for survivability but they may start to die after plating. The quality of the EBs, NPCs, and neurons highly depend on the starting mESCs. mESCs that have been passaged for too many times and kept in culture for more than 1 week typically start to lose pluripotency and may not successfully undergo differentiation. Thus, maintaining the mESCs in an optimal condition is key in ensuring that they can differentiate into EBs, NPCs, and neurons.

Minor Concerns:

18. The manuscript could benefit from some editing for grammar, missing words and subject-verb agreement, etc. It is recommended that authors delete irrelevant "general" phrases and sentences, repeated and unneeded words. They should use short sentences. It is also recommended that authors send their manuscript to an expert in English editing and academic writing. For instance, this sentence requires revision and adding semicolon before therefore and comma after it: "Also, the traditional assays to evaluate differentiation efficiency rely on qualitative examination on selected marker genes therefore lack robustness and fail to grasp global changes in gene expression"

    Response: We thank you for paying close attention to this matter. We have made the appropriate edits and we have sent our revised manuscript to our colleagues in academic writing for review.

19. Please revise all figure legends and make them informative of the test/methodology used and main results presented. Include the statistical test used, mean ± SD or SEM, etc.

    Response: We have revised our figure legends where we added more information on the method used for each figure and the appropriate statistical test used (mean ± SD was used in the RT-qPCR experiment).

Reviewer #2:

1. This paper by Gao and colleagues provides a step-by-step protocol for differentiation of ESCs to neurons and outlines some basic molecular characterizations. While the protocol is detailed and nicely written, it is not any different or unique than the standard protocols used in many labs. Moreover, some steps are not well expanded upon. The procedures of RNA isolation and cDNA synthesis and RT-qPCR are standard and do not need to be part of this protocol in detail. I think this protocol would benefit from going into detail about the differentiation steps. Following are some comments that can improve the protocol.

Response: We appreciate the reviewer for the comments and suggestion. Our intention is to present a comprehensive analysis that can be applied to evaluate the neuronal differentiation process rather than introducing any novel methods. We agree that some of the procedures such as RNA isolation, cDNA synthesis, and RT-qPCR can be compressed so that the differentiation steps can be further elaborated.

2. NPC to neuron differentiation gives mixed cultures (astrocytes and oligodendrocyte) this should be commented on and accounted for in the protocol.

   Response: Thank you for bringing this up. Indeed, we have included further explanation that NPC differentiation using RA does result in mixed neural cell cultures. Additionally, we discussed that the NPC population can be probed for neuron and other neural lineage markers such as *Gfap* for astrocytes and *Olig2* for oligodendrocytes to check the culture's heterogeneity via RT-qPCR or immunofluorescence.

3. ESC make EBs, and then EBs make NPCs. The NPCs can be passaged and frozen and thawed and characterized as stable multipotent lines. This can be included.

   Response: This is an excellent point. Although we did not freeze our NPCs, they can certainly be frozen and cultured again at a later time. We have included this in the text.

4. EBs and NPCs terms should not be used interchangeably.

   Response: We reviewed our manuscript and made necessary changes.

5. Mention time of incubation at all steps (i.e 5mins for trypsinization at 37C).

   Response: Thank you for noticing. We have included the trypsin incubation time of 3 minutes in the protocol.

6. Acutase seems to work better for NPC passaging (perhaps mention of it)

   Response: Thank you for bringing this to our attention. We do not use Acutase in our laboratory hence we cannot comment on how well it will work. We mentioned that Acutase can be used as an alternative to trypsin when passaging NPCs.

7. Pre-plating ESCs on gelatin for 45min can get rid of feeders efficiently instead of serial passaging on gelatin several times to get rid of feeders. Continued culturing of ESCs on gelatin affects their differentiation potential.

   Response: This is a good point, thank you for the recommendation.

8. Refer to feeders as feeders or irradiated MEFs, not just as MEFs

Response: We have changed MEFs into irradiated MEFs.

9. Mention concentration of each component of each media.

   Response: The concentration of each component is added.

10. Here the 2i/LIF media with serum is used to culture ESCs. Classic mouse ESC media are either 2i/Lif or serum/Lif. They can clarify why they have used the combination of all.

    Response: Thank you for raising this point. We are aware that there are various ways to culture mESCs. In our case, we have found that omitting serum from the medium can cause the mESCs to die while mESCs cultured in medium in the absence of 2i tend to differentiate more spontaneously. mESCs cultured in 2i/LIF with serum do not show apparent cell death and they tend to form more single spherical colonies.

    References:

    1. Wang, Qiang, et al. "WDR68 Is Essential for the Transcriptional Activation of the PRC1-AUTS2 Complex and Neuronal Differentiation of Mouse Embryonic Stem Cells." *Stem Cell Research*, vol. 33, 12 Nov. 2018, pp. 206–214., doi:10.1016/j.scr.2018.10.023.
    2. Sim, Ye-Ji, et al. "2i Maintains a Naive Ground State in ESCs through Two Distinct Epigenetic Mechanisms." *Stem Cell Reports*, vol. 8, no. 5, 9 May 2017, pp. 1312–1328., doi:10.1016/j.stemcr.2017.04.001.

Assays mostly not necessary:

11. AP staining not relevant to this protocol. E14 line is a very established pluripotent line and there is no need to AP stain them before use as long as they are cultured well. Plus AP staining is not a solid marker of pluripotency.

    Response: We appreciate this comment. Indeed, E14 is an established cell line but we think that it is no harm to include AP staining as an assessment for the mESCs. We agree that AP activity is not the best marker for pluripotency and we do provide other means to verify the mESCs' pluripotency markers (Nanog and Oct4) using RT-qPCR.

12. For RNA isolation a kit should be used (like Qaigen). Trizol based reagents interferes with most RNAseq library preps and is not recommended by many next gen sequencing cores. Is TriPure Trizol based?

    Response: We appreciate your concern. TriPure is indeed TRizol-based. We have used the same RNA extraction method for other RNA-seq experiments and we have not encountered major problems with it.

13. cDNA and RT-qPCR protocols are standard. Just refer to the superscript kit and SYBR green kit (this is standard and unnecessary to put as step by step here)

Response: Thank you for bringing this up. They are indeed standard procedures and they are now condensed.

14. I.F. procedures are relevant. NPCs should be stained, and neurons as well. Comment on other cell types that may arise here. Triton X 1000 is used (is it a typo? as mostly TritonX 100 is used).

    Response: We stained our NPCs at day 8 (before plating) with Nestin antibody and neurons with Neurofilament antibody (Figure 3A). In the discussion, we further comment that other cell types such as astrocytes and oligodendrocytes can arise from NPC differentiation and that this can be verified with RT-qPCR or IF. It is indeed a typo, we used Triton X-100. Thank you for noticing.

15. RNAseq is irrelevant as once you have RNA isolated, all RNAseq analyses are the same. But it is good that they have kept it concise referring to and previous detailed protocol.

    Response: We agree that RNA-seq analyses are mostly similar and we have condensed the procedure for this step.

16. The use of reporter line and FACS is nice. The significance of sox1 reporter should be explained. Why is it a good reporter for neurons? Comment on its limitation that it can only be done in lines that have a reporter and not routinely on any cell line.

    Response: Thank you for pointing this out. We included in the discussion that Sox1 is one of the earliest markers specifically expressed during neural lineage specification hence it is a good reporter for neurons. We are now mentioning that indeed our FACS analysis is for the Sox1-GFP reporter line and that it cannot be conducted in any other mESC line. The FACS analysis was conducted to test how well our neuron differentiation method is. We can still quantify Sox1 expression with RT-qPCR or Western blot.

Figures:

17. Figure 1: Images are yellowish, should be gray, or black and white bright field.

    Response: Thank you for your concern. We have changed the color shade to black and white.

18. Figure 2: The flow chart is nice. Perhaps a nicer picture of NPCs and Neurons would have been useful.

    Response: We thank the reviewer for this suggestion. However, given the current pandemic situation in the US including where our school is located, we are unable to perform experiments to obtain new pictures.

19. Figure 3: Panel A is an EB that contains NPCs, please refer to as such.

    Response: Thank you for pointing this out. This is true and we have made the appropriate change.

Reviewer #3:

This manuscript nicely described a step-by-step procedure for differentiating mouse embryonic stem cells into neural progenitors and neurons, together with a series of rigorous assays to characterize the differentiated cells. This is a much-needed protocol that will be of tremendous interest to scientists in related fields. The manuscript is well written and carefully prepared. Quick publication is strongly recommended.

Response: We appreciate the reviewer for the positive comments.

Response to Editors for Manuscript Entitled "Differentiation and characterization of neural progenitors and neurons from mouse embryonic stem cells"

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

   Response: We have reviewed the manuscript and corrected any spelling or grammatical errors.

2. **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

   Response: We have ensured that the highlighted text in our protocol can be filmed coherently.

3. **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

   1) Some of your shorter protocol steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

      Response: Where relevant, we have combined actions that can be mentioned in the same step.

   2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

      Response: We thank you for this reminder. We have reviewed our highlighted protocol again and ensured that the highlighted steps are coherent.

   3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

      Response: We have ensured that we highlighted the relevant text, which sums up to 2 pages including sub-headings and spaces.

   4) Notes cannot be filmed and should be excluded from highlighting.

Response: We have removed the highlighted notes in the protocol section.

4. **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs):

    1) Modifications and troubleshooting

    Response: We have discussed the appropriate modifications and troubleshooting needed to be done in the protocol. For example, we stressed that starting with optimally pluripotent mESCs is critical in ensuring that the EB, NPC, and neuron differentiation can occur effectively. Here, experiments such as alkaline phosphatase (AP) and RT-qPCR discussed in the manuscript can determine of the starting mESCs are in the best pluripotent condition.

    2) Limitations of the technique

    Response: We have further expanded the limitations to our methods. This included the limitations of *in vitro* assays, the limited number of cell line used, and the problems that come with using retinoic acid and neuronal cell culture supplements.

    3) Significance with respect to existing methods

    Response: We have clarified that the methods we present comprehensively analyze the differentiation process of mESCs into EBs, NPCs, and neurons. We have mentioned that our set of assays can identify specific genes or cellular pathways related to mESC differentiation.

    4) Future applications

    Response: We mentioned that the methods we presented theoretically can be applied to other embryonic stem cell lines. With further optimization, these methods can be combined with techniques such as 3D cultures or organoids to better mimic the physiological conditions.

    5) Critical steps within the protocol.

    Response: We have stressed in the discussion section the critical steps in the protocol. For example, we mentioned that maintaining the mESCs in the most optimal ESC condition is vital in ensuring that the differentiation process can proceed effectively.

5. **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of

commercial sounding language in your manuscript are Stemgent, Eppendorf, (Invitrogen, Cat. # 15596026, TriPure, e SuperScript™ III R, etc

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

   Response: We have removed all commercial names of the reagents/equipment mentioned in the manuscript and replaced with more general names.

2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

   Response: We have removed all product names containing the trademark symbols.

6. If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

   Response: We thank you for the reminder. The figures and tables that we have submitted have not been published elsewhere.