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TITLE:

Neuronal Differentiation from Mouse Embryonic Stem Cells In vitro

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KEYWORDS:

Mouse embryonic stem cells, neural differentiation, embryoid body, retinoic acid, N2B27 medium

SUMMARY:

Here, we established a low cost and easy to operate method that directs fast and efficient differentiation from embryonic stem cells into neurons. This method is suitable for popularization among laboratories and can be a useful tool for neurological research.

ABSTRACT:

The neural differentiation of mouse embryonic stem cells (mESCs) is a potential tool for elucidating the key mechanisms involved in neurogenesis and potentially aid in regenerative medicine. Here, we established an efficient and low cost method for neuronal differentiation from mESCs in vitro, using the strategy of combinatorial screening. Under the conditions defined here, the 2-day embryoid body formation + 6-day retinoic acid induction protocol permits fast and efficient differentiation from mESCs into neural precursor cells (NPCs), as seen by the formation of well-stacked and neurite-like A2lox and 129 derivatives that are Nestin positive. The healthy state of embryoid bodies and the timepoint at which retinoic acid (RA) is applied, as well as the RA concentrations, are critical in the process. In the subsequent differentiation from NPCs into neurons, N2B27 medium II (supplemented by Neurobasal medium) could better support the long term maintenance and maturation of neuronal cells. The presented method is highly efficiency, low cost and easy to operate, and can be a powerful tool for neurobiology and developmental biology research.

INTRODUCTION:

Embryonic stem cells (ESCs) are pluripotent and can differentiate into neural precursor cells

(NPCs) and subsequently into neurons under certain conditions[1]. ESC-based neurogenesis provides the best platform to mimic neurogenesis, thus serving as a useful tool for developmental biology studies and potentially aid in regenerative medicine[2-3]. In the past decades, many strategies have been reported for inducing embryonic neurogenesis, such as the transgenic method[4], using small molecules[5], using a 3D matrix microenvironment[6], and the co-culture technique[7]. However, most of these protocols are either condition limited or hard to operate, thus they are not suitable for usage in most laboratories.

To find an easy to operate and low cost method to achieve efficient neural differentiation from mESCs, a combinatorial screening strategy was used here. As described in **Figure 1**, the whole process of embryonic neurogenesis was divided into 2 phases. Phase I refers to the differentiation process from mESCs into NPCs, and phase II relates to the subsequent differentiation from NPCs into neurons. Based on the principles of easy operation, low cost, easily available materials and high differentiation efficiency, seven protocols in Phase I and three protocols in Phase II were chosen based on the traditional adherent monolayer culture system or embryoid body formation system[8-9]. The differentiation efficiency of protocols in both phases was evaluated using cell morphology observation and immunofluorescence assay. Through combining the most efficient protocol of each phase, we established the optimized method for neural differentiation from mESCs.

PROTOCOL:

1. Mouse embryonic stem cell culture

1.1. Prepare 0.1% gelatin coated cell culture dishes or plates.

1.1.1. Add 2 mL of sterilized 0.1% gelatin (0.1% w/v in water) to 60 mm cell culture dishes. Rock gently to ensure even coating of the cell culture dishes.

1.1.2. Put the dishes into a 5% CO_2 incubator at 37 °C and allow coating for 1 h.

1.1.3. Remove the 0.1% gelatin solution before seeding the cells.

NOTE: After removing the gelatin, there is no need to dry or wash the coated dishes.

1.2. Mouse embryonic stem cells (A2lox and 129) culture

1.2.1. Incubate mESCs (A2lox and 129) cells in the 0.1% gelatin coated 60 mm cell culture dishes in mESC growth medium at 37 °C in a 5% CO_2 incubator, respectively. The mESC growth medium consists of 85% knock-out DMEM/F12, 15% Knock-out serum replacement (KSR), 0.1 mM β-mercaptoethanol (2ME), 2 mM GlutaMAX, 1% non-essential amino acid (NEAA), 1% penicillin/streptomycin (P/S), 1000 U/mL leukemia inhibitory factor (LIF), 10 nM CHIR-99021 (GSK-3 inhibitor) and 0.33 nM PD0325901 (MEK inhibitor).

CAUTION: β-mercaptoethanol is flammable and has inhalation toxicity. Keep away from fire sources and wear a mask to avoid inhalation when use.

1.2.2. Change the mESC growth medium daily for better growth of A2lox and 129.

1.2.3. When the cells reach 80% confluence, remove the medium and add 1 mL of 0.1% trypsin to the dish. Gently rock for 30 s to ensure even cover of trypsin on all cells.

1.2.4. Leave the cells for about 1 min to trypsinize and then remove the trypsin using 1 mL pipette.

1.2.5. Add 2 mL of mESC growth medium to the dish, pipette up and down several times to make a single cell suspension.

1.2.6. Count the density of the cells in the suspension as accurately as possible using hemocytometer.

1.2.7. Divide the cells into 7 groups and induce differentiation using different protocols shown in **Table 1**.

2. Differentiation from mESCs to NPCs (Phase I)

2.1 Prepare 0.1% gelatin coated cell culture plates or coverslips.

2.1.1. Before use, prepare 0.1% gelatin coated 6-well plates or coverslips as in step 1.1.

2.2. Phase I differentiation using protocol 1 (**Table 1**)

2.2.1. Seed about 2 x 10^4 mESCs in 2 mL of basal differentiation medium I per well in the 0.1% gelatin-coated 6-well plates. Check the cell density under a microscope.

2.2.2. Incubate the cells at 37 °C in a 5% CO_2 incubator for 6 h to allow for attachment.

2.2.3. After attachment, take out of the cells from incubator, and wash the cells twice with 2 mL of PBS.

2.2.4. Add 2 mL of basal differentiation medium I (**Table 1**) to each well and put the cells back into the incubator.

2.2.5. Leave the cells for differentiation for 8 days. Replace the basal differentiation medium I every 2 days.

2.3. Phase I differentiation using protocol 2 (**Table 1**)

2.3.1. Add 1.5 x 10^6 mESCs into a nonadhesive bacterial dish in 10 mL of basal differentiation

medium I to allow for embryoid body formation at 37 °C in a 5% CO_2 incubator.

2.3.2. After 2 days, transfer cell aggregates into 15 mL centrifuge tubes and let them settle by gravity.

2.3.3. Remove the supernatant and add 10 mL of fresh basal differentiation medium I to resuspend the embryoid bodies. Replant them into a new nonadhesive bacterial dish and allow differentiation for another 2 days.

2.3.4. Check the formation of embryoid bodies under the microscope (**Figure 2A**).

2.3.5. Collect embryoid bodies as described in steps 2.3.2-2.3.3. Seed about 50 embryoid bodies in 2 mL of basal differentiation medium I per well onto 0.1% gelatin-coated 6-well plates.

2.3.6. Prepare 1 mM all-trans RA stock (in DMSO) and store away from light in a -80 °C freezer after sub-packaging.

NOTE: RA is unstable, and attention should be paid to keeping it away from light and reducing air contact during preparation of RA stock.

2.3.7. For RA induction, add 2 µL of RA stock into each well to make a final concentration of 1 µM.

2.3.8. Place the plate into the 5% CO_2 incubator at 37 °C and differentiate for another 4 days.

2.3.9 Change the entire 2 mL of basal differentiation medium I (with 1 µM RA) every 2 days.

2.4. Phase I differentiation using protocol 3 (**Table 1**)

2.4.1. Plant 1.5 x 10^6 mESCs into a nonadhesive bacterial dish in 10 mL of basal differentiation medium I. Leave for 2 days for embryoid body formation at 37 °C in a 5% CO_2 incubator.

2.4.2. Check the formation of embryoid bodies under a microscope (**Figure 2B**).

2.4.3. Transfer cell aggregates into 15 mL centrifuge tubes and let them settle by gravity.

2.4.4. Remove the supernatant carefully and add 10 mL of fresh basal differentiation medium I to resuspend them.

2.4.5. Seed about 50 embryoid bodies into 2 mL of basal differentiation medium I per well onto 0.1% gelatin-coated 6-well plates.

2.4.6. For RA induction, add 2 µL of RA stock into each well to make a final concentration of 1 µM.

2.4.7. Place the plate into the 5% CO_2 incubator at 37 °C and differentiate for another 6 days. Change the entire basal differentiation medium I (with 1 μM RA) every 2 days.

2.5. Phase I differentiation using protocol 4 (**Table 1**)

2.5.1. Seed about 2 x 10^4 mESCs within 2 mL of basal differentiation medium I per well onto the 0.1% gelatin-coated 6-well plates. Place the plate into the 5% CO_2 incubator at 37 °C to allow for attachment for 6 h.

2.5.2. After attachment, wash the cells twice with 2 mL of PBS. Add 2 mL of basal differentiation medium I to each well and allow for differentiation for 4 days in the 5% CO_2 incubator at 37 °C.

2.5.3. For RA induction, add 2 μL of all-trans RA stock into each well (the working concentration is 1 μM) to induce differentiation for another 4 days.

2.5.4. In the whole process, replace the entire medium every 2 days.

2.6. Phase I differentiation using protocol 5 (**Table 1**)

2.6.1. Seed about 2 x 10^4 mESCs within 2 mL of basal differentiation medium I per well onto the 0.1% gelatin-coated 6-well plates. Place the plate into the 5% CO_2 incubator at 37 °C to allow for attachment for 6 h.

2.6.2. Wash the cells twice with 2 mL of PBS. Add 2 mL of basal differentiation medium I to each well and allow for differentiation for 2 days in the 5% CO_2 incubator at 37 °C.

2.6.3. For the subsequent RA induction, add 2 μL of RA stock into each well to make a final concentration of 1 μM. Place the plate into the 5% CO_2 incubator at 37 °C to induce differentiation for another 6 days.

2.6.4. In the whole process, replace the entire medium every 2 days.

2.7. Phase I differentiation using protocol 6 (**Table 1**)

2.7.1 Plant 1.5 x 10^6 mESCs into a nonadhesive bacterial dish in 10 mL of N2B27 medium II (**Table 1**) to allow for embryoid bodies formation.

2.7.2. On the 2nd day, collect the cell aggregates as described in steps 2.3.2-2.3.3 and resuspend the embryoid bodies using 10 mL of fresh N2B27 medium II.

2.7.3. Replant them into a new nonadhesive bacterial dish and allow differentiation for another 2 days in the 5% CO_2 incubator at 37 °C. Check the formation of embryoid bodies under microscope.

2.7.4. On the 4th day, collect embryoid bodies. Seed about 50 embryoid bodies per well onto 0.1% gelatin-coated 6-well plates with 2 mL of N2B27 medium II.

2.7.5. Add 2 μL of all-trans RA stock into each well and induce differentiation for another 4 days. Replace the entire medium (N2B27 medium II with 1 μM RA) every two days.

2.8. Phase I differentiation using protocol 7 (**Table 1**)

2.8.1. Seed about 2 x 10^4 mESCs within 2 mL of basal differentiation medium I per well onto the 0.1% gelatin-coated 6-well plates. Place the plate into the 5% CO_2 incubator at 37 °C to allow for attachment for 6 h.

2.8.2. Wash the cells twice with PBS. Then, add 2 mL of N2B27 medium II to each well and allow for differentiation for 8 days at 37 °C in a 5% CO_2 incubator.

2.8.3. Change the entire N2B27 medium II every 2 days.

3. Cell morphology observation

3.1. Check the differentiation status of the above-mentioned 7 groups daily under an inverted phase contrast light microscope.

3.2. Randomly select at least 12 fields and take photos to record the morphological changes of each group on D8.

4. Immunofluorescence staining

4.1. Sample preparation: Seed mESCs on 0.1% gelatin-coated coverslips and allow for differentiation for 8 days using the protocols mentioned in step 2.

4.2. Rinse: On the 8th day, take the samples out from the incubator and remove the differentiation medium by aspiration. Gently rinse the cells once with 1 mL of PBS for 5 min.

4.3. Fixation: Add 1 mL of 4% paraformaldehyde to each sample and fix the cells for 20 min at room temperature (RT).

4.4. Rinse: After fixation, gently rinse the cells with 1 mL of PBS 3 times, for 5 min each.

4.5. Permeabilization: Add 1 mL of 0.2% TritonX-100 in PBS to each sample and leave for 8 min at RT.

4.6. Rinse: After permeabilization, gently rinse the cells with 1 mL of PBS 3 times, for 5 min each.

4.7. Blocking: Add 1 mL of 10% goat serum in PBS to each sample and incubate at RT for 1 h to block any non-specific interactions.

4.8. Incubation with primary antibody

4.8.1. Dilute the anti-Nestin antibody at a ratio of 1:100 using 5% goat serum in PBS.

4.8.2. Apply 500 µL of diluted antibody to different samples and incubate overnight at 4 °C.

4.9. Rinse: Remove the antibody and rinse the samples gently with 1 mL of PBS 3 times for 8 min each.

4.10. Incubation with secondary antibody

4.10.1. Dilute the Alexa Fluor 488-labeled goat anti-mouse IgG at a ratio of 1:500 using 5% goat serum in PBS.

4.10.2. Apply 500 µL of diluted antibody to different samples and incubate in dark for 2 h at RT.

NOTE: After applying fluorescent secondary antibody, perform all the subsequent steps in the dark to prevent fluorescence quenching.

4.11. Rinse: Remove the secondary antibody and rinse the samples gently with 1 mL of PBS 3 times for 8 min each.

4.12. Nuclear staining and mounting

4.12.1. Place one drop of DAPI mounting medium onto the clean microslide.

4.12.2. Carefully take out of the samples from the plates and place the sample on top of the DAPI mounting medium with the cell face down. Leave in the dark for 5 min at RT.

4.12.3. Remove excess DAPI mounting medium with absorbent paper.

4.13. Fluorescence microscopy observation

4.13.1. Place the specimens under the fluorescence microscopy and detect the signal for DAPI and Alexa Fluor 488 using proper filters.

4.13.2. Evenly and randomly pick 10-15 different visual fields for each sample and record the images with a CCD camera.

5. Differentiation from NPCs to neurons (Phase II)

5.1. Prepare mESC derivatives under Phase I differentiation using protocol 3 (8 days, **Table 1**) as detailed in step 2.4, which has the highest differentiation efficiency (See **Figure 3**).

NOTE: After phase I differentiation, quality control should be carried out using cell morphology observation and immunofluorescence assay mentioned above, to ensure a healthy and high-yield NPCs.

5.2. Seed about 5 x 10^5 mESC derivatives within 2 mL of basal differentiation medium I per well onto the 0.1% gelatin-coated 6-well plates. Randomly divide the mESC derivatives into 3 groups, as Phase II protocol 1, protocol 2, and protocol 3, respectively.

5.3. Place the plate into the 5% CO_2 incubator at 37 °C to allow for attachment for 6 h. Wash them twice with 2 mL of PBS.

5.4. Add 2 mL of basal differentiation medium I, N2B27 medium I and N2B27 medium II (**Table 2**), respectively, to each well of the above groups.

5.5. Place the plates into the incubator and allow to differentiate for another 10 days. Change the corresponding medium every 2 days.

5.6. Check the differentiation status and record the morphological changes as mentioned in step 3.

5.7. On Day 18, evaluate the generation of neurons (β-Tubulin III positive) and determine the differentiation efficiency of the 3 protocols using in step 4.

REPRESENTATIVE RESULTS:

2-day embryoid body formation + 6-day RA induction works best on directing the differentiation of mESCs into NPCs (Phase I). To determine the optimal protocol that best promote the differentiation of mESCs into NPCs (Phase I), 7 protocols were tested on both A2lox and 129 mESCs (**Table 1**) and the differentiation status of each group was monitored using light microscope. As shown in **Figure 3A**, most A2lox and 129 derivatives under "2-day embryoid body formation + 6-day RA induction" treatment (Phase I-protocol 3) showed well-stacked and neurite-like morphologies, which indicating the formation of NPCs. However, cells with "4-day embryoid body formation + 4-day RA induction" treatment (Phase I-protocol 2) showed poor and apoptotic status, which may be due to the lack of nutrient within embryoid bodies. Monolayer culture combined with RA induction (Phase I-protocol 4 and 5) could also direct the differentiation of mESCs, while the proportion of neurite-like cells was not as much as that in Phase I-protocol 3. Meanwhile, most A2lox and 129 derivatives in Phase I-protocol 6 and 7 showed smaller cell bodies and tended to undergo apoptosis, suggesting that N2B27 medium II could not support embryonic neurogenesis effectively.

To further confirm the formation of NPCs, the percentage of Nestin+ cells (marker for NPCs) in each group were detected using an immunofluorescence assay. In **Figure 3B**, the percentage of

Nestin+ cells in Phase I-protocol 3 were the highest and reached up to 77.67 ± 4.33% and 69.33 ± 2.33% in A2lox and 129 derivatives, respectively. Collectively, Phase I-protocol 3 works best on directing the differentiation of mESCs into NPCs.

N2B27 medium II can most effectively induce the differentiation from NPCs into neurons (Phase II). Three protocols in phase II differentiation were examined. As shown in **Figure 4A**, morphological observation showed that most A2lox and 129 derivatives in phase II-protocol 3 (differentiation with N2B27 medium II) appeared the most prolonged neuron-like structures with clear neurites and cell body extensions by Day 18, indicating the efficient occurrence of neurogenesis. Immunofluorescence assays further confirmed the generation of neurons, with the percentage of β-Tubulin III+ cells up to 67.75 ± 4.01% and 58.73 ± 7.25%, respectively, in A2lox and 129 derivatives on D18 (**Figure 4B**).

To make it clearer, a schematic diagram of the optimized method for embryonic neurogenesis is shown in **Figure 5**. Briefly, 1.5 x 10^6 mESCs are seeded into a nonadhesive bacterial dish in 10 mL of basal differentiation medium I and allow for embryoid body formation for 2 days. Then, embryoid bodies are collected and planted into the 0.1% gelatin-coated 6-well plates with the concentration of 50 embryoid bodies per well. Meanwhile, RA (1 μM) is added for another 6 days. From Day 8 to Day 18, RA is removed, and N2B27 medium II is applied to direct the subsequent differentiation from NPCs to neurons. With such a combined method, robust neurons can be formed on Day 18.

FIGURE AND TABLE LEGENDS:

Figure 1: Diagram of the embryonic neurogenesis process. This figure has been modified from Li et al.[10].

Figure 2: The morphology of the embryoid bodies. (**A**) Embryoid bodies cultured for 4 days. (**B**) Embryoid bodies cultured for 2 days.

Figure 3: Efficiency comparison of the 7 protocols on phase I differentiation using A2lox and 129 mESCs. (**A**) Morphological analysis of A2lox and 129 mESCs derivatives on Day 8. Upper panel: A2lox derivatives; Lower panel: 129 derivatives. (**B**) Immunofluorescence detection for the formation of NPCs (Nestin+, green). The nuclei were labeled blue with DAPI. Upper panel: A2lox derivatives on D8; Lower panel: 129 derivatives on D8. Percentages of Nestin+ cells of each group were shown by histogram. Each *column* represents the mean±SEM of three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$. This figure has been modified from Li et al.[10].

Figure 4: Efficiency comparison of the 3 protocols on phase II differentiation. (**A**) Morphological analysis of A2lox and 129 mESCs derivatives on Day 18. Upper panel: A2lox derivatives; Lower panel: 129 derivatives. (**B**) Immunofluorescence detection for the formation of neurons (β-Tubulin III+, red). The nuclei were labeled blue with DAPI. Upper panel: A2lox derivatives on D18; Lower panel: 129 derivatives on D18. Percentages of β-Tubulin III+ cells of each group were shown by histogram. Each *column* represents the mean ± SEM of three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$. This figure has been modified from Li et al.[10].

Figure 5: Brief model of the optimized method for neuronal differentiation from mESCs in vitro. This figure has been modified from Liet al.[10].

Table 1: Details of the 7 protocols used in phase I differentiation. This table has been modified from Li et al.[10].

Table 2: Details of the 3 protocols used in phase II differentiation. This table has been modified from Li et al.[10].

DISCUSSION:

In the present study, we established a simple and effective method for neuronal differentiation from mESCs, with low cost and easily obtained materials. In this method, 2 days of embryoid body formation followed by 6 days of RA induction can effectively promote the differentiation of mESCs into NPCs (Phase I-protocol 3). For the phase II differentiation, N2B27 medium II (Phase II-protocol 3) most effectively induce the differentiation from NPCs into neurons. To ensure success, more attention should be paid to several critical steps.

Firstly, the healthy state of embryoid bodies is the key for the whole differentiation process. Three-dimensional embryoid body formation is usually used to direct the differentiation of ESCs[8]. In this study, we investigated the proper suspension culture time of embryoid bodies. As shown in **Figure 2**, round embryoid bodies with bright cores were formed after suspension culture for 2 days in this condition. However, when cultured for 4 days, many embryoid bodies adhere to each other, and the cores become dark, indicating the apoptosis of cells in the cores. The subsequent differentiation further confirmed the worse effect of prolonged embryoid body formation. In some reported studies, suspension culture of embryoid bodies could last for as long as 10 days, using medium with lower FBS concentration or without FBS[11]. The reduced time for embryoid body formation in the study may be due to the higher FBS concentration (15%) used here, and it has been proven that 15% FBS can better promote the formation and differentiation of embryoid bodies.

Secondly, the timepoint at which RA is applied and the RA working concentration are critical for cell fate determination of mESCs. RA, a derivative of vitamin A, is one of the most important morphogens with pleiotropic actions[12]. RA can regulate multiple signal pathways and affect cell fate determination of ESCs[13-14]. Reports showed that short-term treatment of mESCs with RA during the early differentiation stage prevented spontaneous differentiation and maintain self-renewal capacity of mESCs[15]. Others suggested that RA could regulate both germ cell differentiation and neural differentiation from ESCs, which are timepoint dependent[16-18]. In the condition presented here, RA added on the 2nd day after embryoid body formation is appropriate for directing the differentiation into NPCs. Meanwhile, the working concentration of RA is also critical. Low RA concentrations (~10 nM) may induce the differentiation of mESCs into endoderm-like cells, whereas high RA concentrations (1-5 μM) are more likely to induce differentiation into NPCs[13-19]. Due to the use of RA, one would expect a caudalization effect; the differentiation into fore brain neurons would be rarely seen and yielding neurons of hindbrain and spinal cord fates

would occur[20-21]. Furthermore, RA is an easily available and low cost agent, and the use of this protocol can save research funds for most laboratory.

Thirdly, in condition presented here, the NPCs generated after phase I differentiation can be stored and passaged in proper conditions. Cryopreservation with high cell density (>2 x 10^6) using Stem-Cellbanker can effectively reduce the cell damage caused by freezing to get a high recovery rate. Meanwhile, NPCs generated in the study can be passaged using N2B27 medium (49% DMEM/F12+ 1% N2 + 48% Neurobasal medium + 2% B27) with a cell density more than 5 x $10^5/cm^2$. Under low cell density (less than 0.5 x $10^5/cm^2$), NPCs tend to differentiate. Such cell cryopreservation and recovery can bring great convenience to the research.

Moreover, Neurobasal medium is essential for the phase II differentiation. Neurobasal medium is designed specifically for long-term maintenance and maturation of neuronal cell. As listed in N2B27 medium II (**Table 2**), the addition of Neurobasal medium could better support the differentiation from NPCs into neurons.

Collectively, we reported an efficient and low-cost method for neuronal differentiation from mESCs in vitro, using the strategies of combinatorial screening. The established method is very easy to implement and is suitable for use by most laboratories. Such an optimized method can be a powerful tool for neurobiology and developmental biology research.

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DISCLOSURES:

The authors have nothing to disclose.

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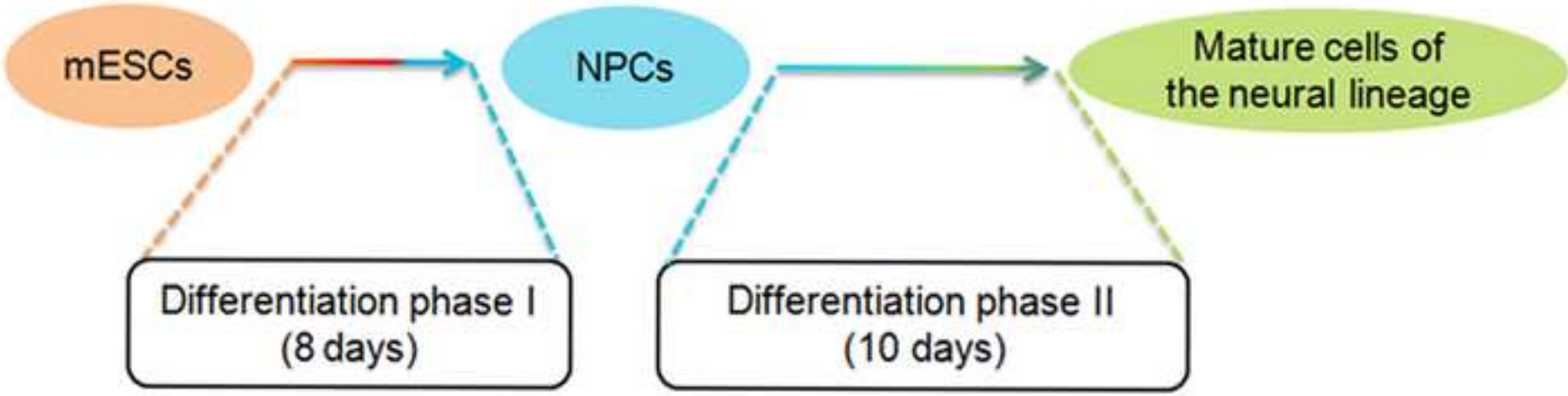

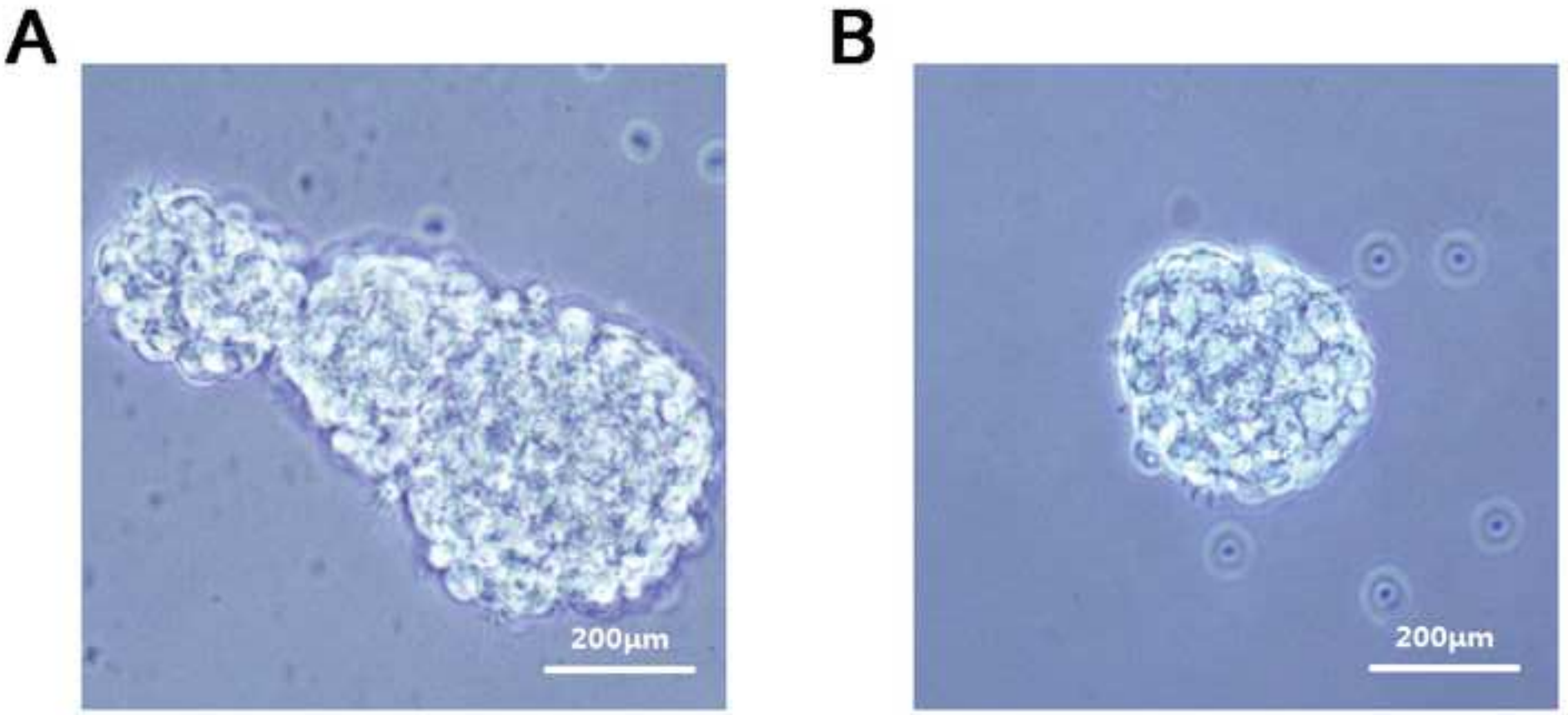

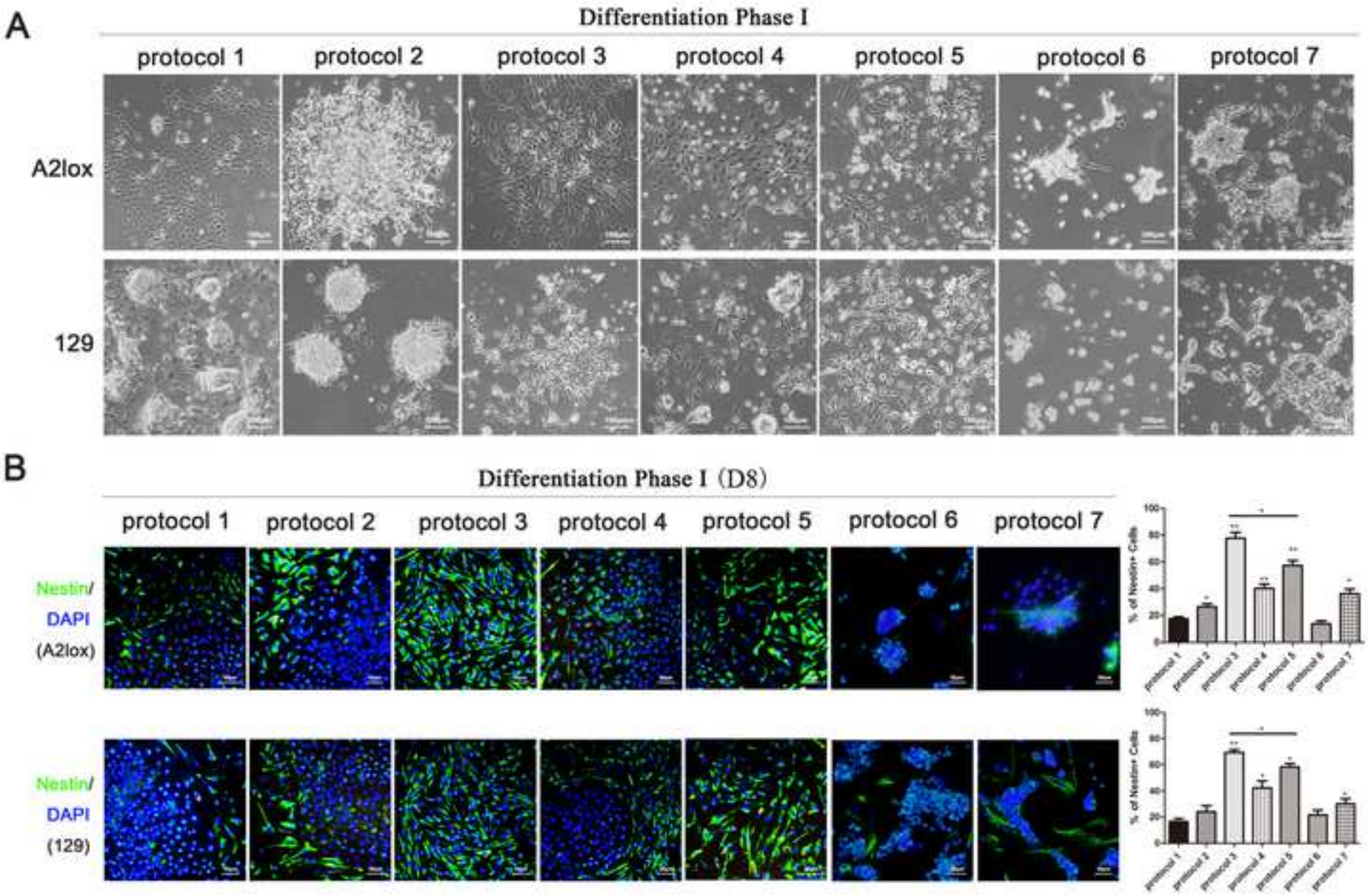

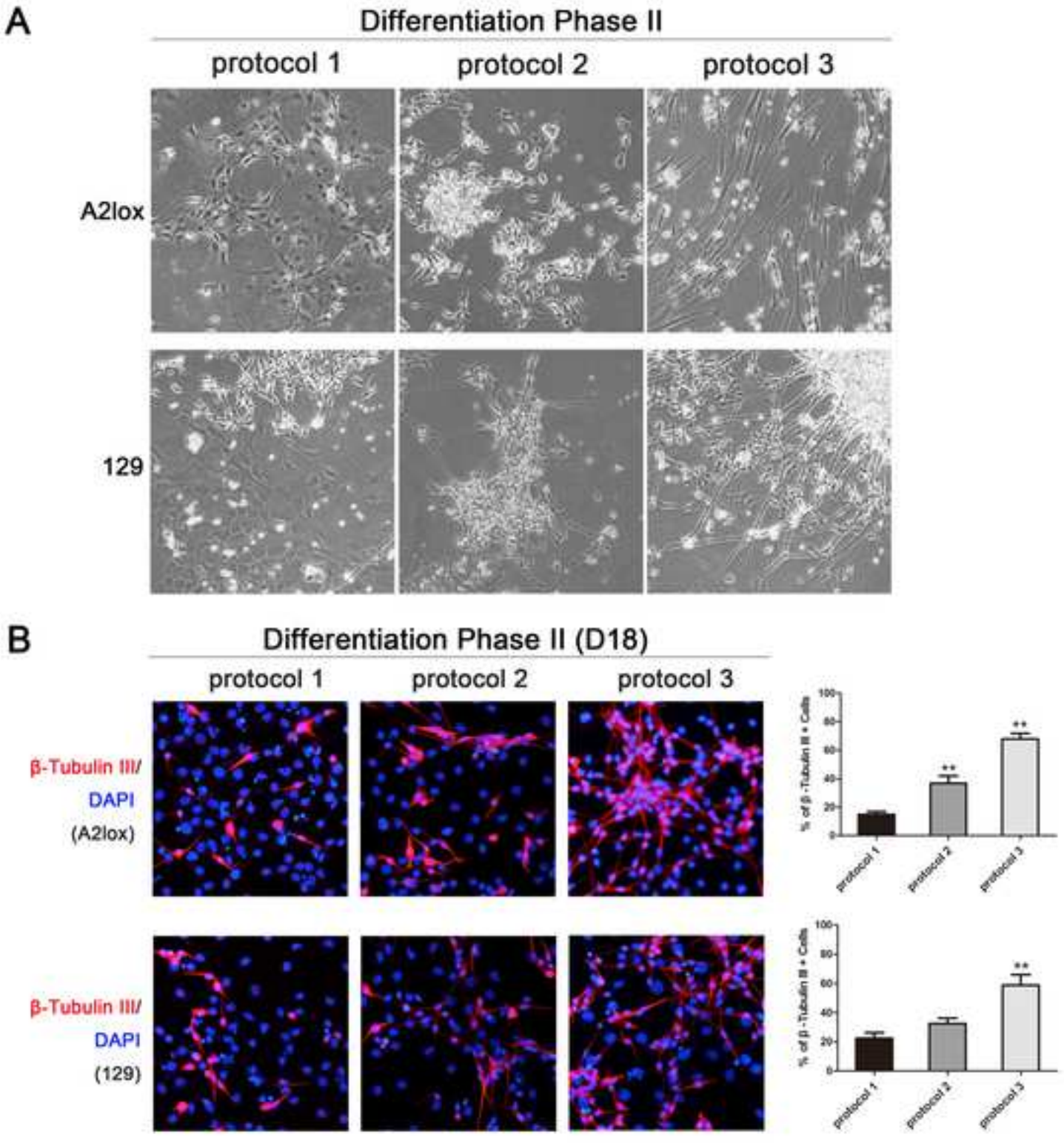

Revised Figure 5

Click here to access/download;Figure;R1-Figure 5.psd

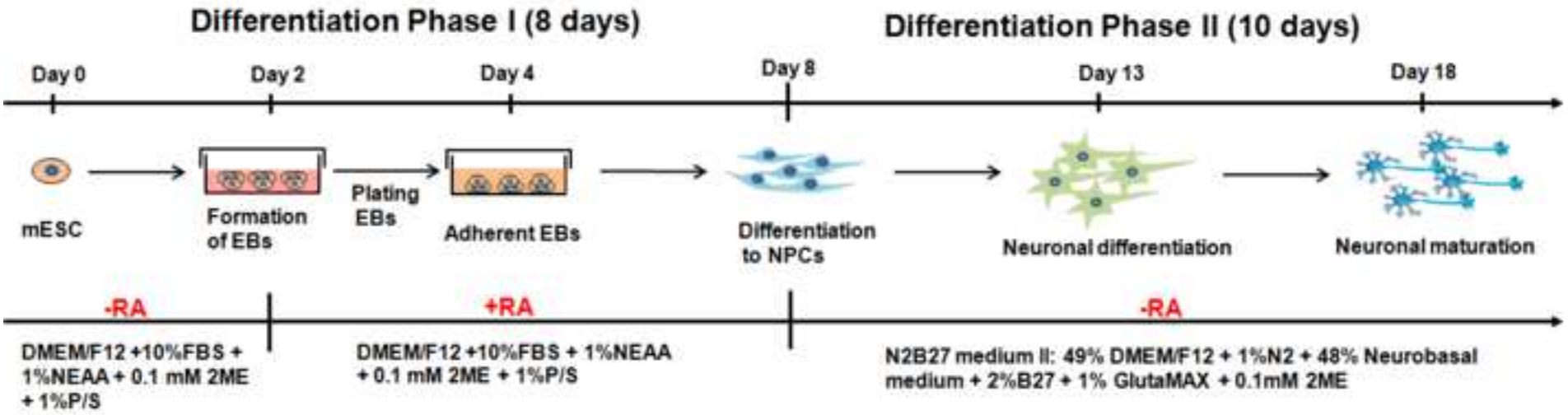

Table 1

Differentiation Phase I (8d)		
	Protocols	Media
protocol 1	Differentiation naturally: With basal differentiation medium I only	Basal differentiation medium I: DMEM/F12 +15%FBS + 1%NEAA+0.1mM 2ME+ 1%P/S
protocol 2	4-day Embryoid Bodies formation + 4-day RA induction	
protocol 3	2-day Embryoid Bodies formation + 6-day RA induction	
protocol 4	Monolayer culture combined with RA induction: 4d (-RA) 4d (+RA)	
protocol 5	Monolayer culture combined with RA induction: 2d (-RA) 6d(+RA)	
protocol 6	Embryoid Bodies formation (4 d) and differentiation induced with N2B27 medium II	N2B27 medium II: 49% DMEM/F12+ 1% N2 + 48% Neurobasal medium + 2% B27 +1%GlutaMAX+ 0.1mM 2ME
protocol 7	Monolayer culture with N2B27 medium II	

Differentiation Phase II (10d)		
	Protocols	Media
protocol 1	Differentiation naturally: With basal differentiation medium I only	Basal differentiation medium I**:** DMEM/F12 +15%FBS +1%NEAA +0.1mM 2ME+ 1%P/S
protocol 2	Differentiation with N2B27 medium I	N2B27 medium I: DMEM/F12 + 1%N2 + 2%B27 + 1%GlutaMAX +0.1mM 2ME
protocol 3	Differentiation with N2B27 medium II	N2B27 medium II: 49% DMEM/F12+ 1% N2 + 48% Neurobasal medium + 2% B27 +1%GlutaMAX+ 0.1mM 2ME

Revised Table of Materials

Click here to access/download;Table of Materials;R1-Table of Materials.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Anti-Nestin antibody [Rat-401]	Abcam	Ab11306	stored at -80 °C, avoid repeated freezing and thawing
Anti-β-Tubulin III antibody produced in rabbit	Sigma Aldrich	T2200	stored at -80 °C, avoid repeated freezing and thawing
Alexa Fluor 488-Labeled Goat Anti-Mouse IgG	Beyotime	A0428	stored at -20 °C and protect from light
B-27 Supplement (50X), serum free	Gibco	17504044	stored at -20 °C, and protect from light
CHIR-99021 (CT99021)	Selleck	S1263	stored at -20 °C
Coverslips	NEST	801007	
Cy3-Labeled Goat Anti-Rabbit IgG	Beyotime	A0516	stored at -20 °C and protect from light
DME/F-12 1:1 (1x)	HyClone	SH30023.01B	stored at 4 °C
Fetal bovine serum	HyClone	SH30084.03	stored at -20 °C, avoid repeated freezing and thawing
Fluorescence microscopy	Olympus	CKX53	
Gelatin	Gibco	CM0635B	stored at room temperature
GlutaMAX Supplement	Gibco	35050061	stored at 4 °C
Immunol Staining Primary Antibody dilution Buffer	Beyotime	P0103	stored at 4 °C
KnockOut DMEM/F-12	Gibco	12660012	stored at 4 °C
KnockOut Serum Replacement	Gibco	10828028	stored at -20 °C, avoid repeated freezing and thawing
Leukemia Inhibitory Factor human	Sigma	L5283	stored at -20 °C
Mounting Medium With DAPI - Aqueous, Fluoroshield	Abcam	ab104139	stored at 4 °C and protect from light
MEM Non-essential amino acids solution	Gibco	11140076	stored at 4 °C

N-2 Supplement (100X)	Gibco	17502048	stored at -20 °C and protect from light
Normal goat serum	Jackson	005-000-121	stored at -20 °C
Neurobasal Medium	Gibco	21103049	stored at 4 °C
Nonadhesive bacterial dish	Corning	3262	
Phosphate Buffered Saline (1X)	HyClone	SH30256.01B	stored at 4 °C
Penicillin/ Streptomycin Solution	HyClone	SV30010	stored at 4 °C
PD0325901(Mirdametinib)	Selleck	S1036	stored at -20 °C
Retinoic acid	Sigma	R2625	stored at -80 °C and protect from light
Strain 129 Mouse Embryonic Stem Cells	Cyagen	MUAES-01001	Maintained in feeder-free culture system
Stem-Cellbanker (DMSO free)	ZENOAQ	stem cellbanker DMSO free	stored at -20 °C, avoid repeated freezing and thawing
Trypsin 0.25% (1X) Solution	HyClone	SH30042.01	stored at 4 °C
Triton X-100	Sigma	T8787	
2-Mercaptoethanol	Gibco	21985023	stored at 4 °C and protect from light
4% paraformaldehyde	Beyotime	P0098	stored at -20 °C
6 - well plate	Corning	3516	
60 mm cell culture dish	Corning	430166	
15 ml centrifuge tube	NUNC	339650	

Dear Editor and reviewers,

We are very grateful to you for yours and reviewers' critical comments and valuable suggestions on our manuscript (**JoVE61190**) entitled "**An efficient and low-cost method for neuronal differentiation from embryonic stem cells in vitro**". Based on these valuable comments and suggestions, we have made careful revision on our original manuscript. All changes made to the manuscript are highlighted in red. We hope the revised manuscript will meet your requirements for acceptance. The followings are point to point responses to the reviewers' comments.

Response to the editor's comments:

Changes to be made by the Author(s):

Q1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

A1. Thank you very much for the comments. We have tried our best to proof-read the manuscript carefully according to your suggestions. All changes made to the manuscript are highlighted in red. We hope the revised manuscript will meet with the requirement for acceptance.

Q2. Please revise the title to remove superfluous words: efficient and low-cost method, etc. One suggestion is: Neuronal differentiation from embryonic stem cells in vitro.

A2. Thank you for your kind suggestions. We fully agree with your comments and have revise the title in our revised manuscript.

Q3. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

A3. Thank you very much for your constructive and valuable comments. We have revised the table of the essential supplies, reagents, and equipment and sorted the

Materials Table alphabetically by the name of the material (see R1-Table of Materials). We hope that the revisions are satisfactory.

Q4. Please add more details to your protocol steps. 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.

A4. Thank you very much for the comments concerning our manuscript. Revisions have been made and all changes made to the manuscript are highlighted in red. We hope that the revisions are satisfactory.

Q5. 1.1.2: Temperature?

A5. Thank you very much for your valuable comments. We have revised the manuscript to make the description of temperature clearer (Line 75). We hope that the revisions are satisfactory.

Q6. 1.2.1: What are the incubation conditions?

A6. Thank you very much for your valuable comments. We have added more details about the incubation conditions (Line 84). We hope that the revisions are clear for the readers to follow.

Q7. 1.2.3: How long are the cells incubated with trypsin?

A7. Thank you very much for your valuable comments. We have added more details about the incubation time with trypsin (Line 96). We hope that the revisions are clear for the readers to follow.

Q8. 1.2.4: How is the trypsin removed? Leave what for 1 min?

A8. Thank you very much for your valuable comments. We have added more details about the actions of trypsinization (Line 98).

Q9. Please specify all incubation conditions.

A9. Thank you for your kind suggestions. We have specified all the incubation condition throughout the PROTOCOL in the manuscript (See Line 121, 134, 156, 163,

177, 183, et al.). We hope that the revisions are satisfactory.

Q10. **Please ensure that the Tables are consistent with the written manuscript. The numbering of the protocols in Table 2 is not consistent with the rest of the text.**

A10. Thank you for pointing out this deficiency. We have revised the numbering of the protocols in Table 2 (see R1-Table2). Special thanks to you for your valuable comments.

Q11. **Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.**

A11. Thank you very much for the comments. We re-identified the essential steps of the protocol for the video and highlighted in yellow with less than 2.75 pages in our revised manuscript. We hope that the revisions are satisfactory.

Q12. **Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.**

A12. Thank you very much for the comments. We have re-identified the highlighted steps according to your suggestions. We hope that the revisions are satisfactory.

Q13. **Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.**

A13. Thank you very much for the comments. We have revised the highlighted steps according to your suggestions. We hope that the revisions are satisfactory.

Q14. **Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the**

editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

A14. Thank you very much for your valuable comments. We have re-obtained an explicit copyright permission to reuse the figures from our previous publication (Li *et al*) and uploaded this information as a .docx file to my Editorial Manager account. Meanwhile, the Figures have been cited appropriately in the Figure Legend (Line 382-383, 407-411). We hope that the revisions are satisfactory.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this manuscript, authors test several protocols for differentiation of neural progenitors cells (NPCs) from mouse embryonic stem cells (mESCs). The authors show that 1 protocol out of 7 has the best efficacy. Then, authors differentiate neurons from NPCs generated using this protocol. Again they evaluate the efficacy of three protocols. In the end, they not only share all 10 (7 for NPC generation and 3 for neuron differentiation) protocols in detail but also present the most effective workflow.

Minor Concerns:

Q1. Caudalization effect of retinoic acid was not discussed. The NPCs generated by use of RA, probably have a caudal identity. Their differentiation into fore brain neurons will be very inefficient or not at all. This is an important limitation of the protocol and should be clear to the readers.

A1. We appreciate your kind suggestions. We have discussed the caudalization effect of RA in our revised manuscript (Line 445-447). We hope that the revisions are clear and unambiguous for the readers.

Q2. Authors do not mention if the NPCs can be passaged and/or stored.

A2. We appreciate your kind suggestions. The NPCs generated in our condition can be

passaged and stored. We have discussed such thing in our revised manuscript (Line 450-456). We hope such revisions can be useful to readers.

Q3. Throughout the manuscript authors do not specify if entire medium was replaced or a percentage of it was replaced during medium changes. It should be clarified.

A3. Thank you very much for your valuable comments. In our protocols, the entire medium was replaced and we have mentioned such information in our revised manuscript (Line 128, 158, 178, 192, 207, 225 and 236). We hope that the revisions are clear and unambiguous for the readers.

Q4. At step 1.1.2, and in general, the temperature of the incubators should be stated.

A4. Thank you very much for your valuable comments. We have stated the temperature of the incubator in the revised manuscript (Line 75).

Q5. At the following steps, authors should specify in which medium the cells were plated/changed and how much medium is used.

1.2.4, 2.2.1, 2.3.5, 2.3.8, 2.4.1, 2.4.5, 2.5.1, 2.5.2, 2.5.3, 2.6.3, 2.7.3

A5. Thank you very much for your valuable comments. More details about the above steps have been given in our revised manuscript (Line 100-101, 118-119, 145-146, 158, 162-163, 172, 182, 186-187, 196, and 221-222). We hope that the revisions are clear and unambiguous for the readers.

Q6. At step 4.5 the permeabilization solution should be described i.e. is Triton in 1x PBS?

A6. Thank you for pointing out this deficiency. A more accurate description has been given in our revised manuscript (Line 260).

Q7. Step 5 needs more details. For example, is the medium change from phase I to phase II complete and sudden (i.e entire medium) or do authors prefer a gradual change. Also it is not clear if authors have a quality control step before proceeding to step 5 after step 4.

A7. Thank you for your valuable comments. More details have been given in Step 5. (Line 312-321). Quality control step after phase I differentiation is very important and we have mentioned this in our revised manuscript (Line 312-314). Special thanks to you for your valuable comments.

Q8. Finally, it is not clear why some text is highlighted in yellow.

A8. I feel very sorry to have troubled you about this. The highlighted text refers to the essential steps of the protocol for the subsequent filming.

Reviewer #2:

Manuscript Summary:

In the manuscript entitled " An efficient and low-cost method for neuronal differentiation from embryonic stem cells in vitro " (JoVE61190), the authors compared the efficiency of seven neural induction protocols and three neuronal differentiation protocols based on traditional neural differentiation systems of mouse ESCs, and selected the most efficient combination of tested protocols. By "combinatorial screening", the authors established an efficient and easy-to-operate method for mouse ESC neural differentiation. The protocols listed in the manuscript were clearly explained and may useful for readers to choose a better differentiation system. But the manuscript can be further improved.

Major concerns:

Q1. Comparing with other published neural differentiation methods, the efficiency (77.67 ± 4.33% and 69.33 ± 2.33% Nestin+ cells in Phase I, 67.75 ± 4.01% and 58.73 ± 7.25% β-Tubulin III+ cells in phase II) of the established protocol in this manuscript seems not very impressively. In protocol 1-5 of differentiation phase I, the authors used the FBS containing medium. BMP in serum inhibits the neural induction of pluripotent stem cells and may reduce the efficiency of neural differentiation. The authors should test other differentiation conditions, such as protocols with EB formation in serum-free cultures (See the SFEB method established by Kiichi Watanabe et al. Nat Neurosci. 2005).

A1. Thank you very much for your constructive and valuable comments. In the study

published by Kiichi Watanabe et al. in Nat Neurosci (2005), treatment with Wnt and Nodal antagonists (Dkk1 and LeftyA) during the first 5 d of SFEB culture causes nearly selective telencephalic precursors differentiation in ES cells (~90%). The high differentiation efficiency can be attributed to the inhibition of Wnt and TGFβ signal pathway, but lead to a selective production of mainly telencephalic precursors. Our study aims to provide researchers an efficient and low-cost method for neuronal differentiation from mESCs *in vitro* without absolute selectivity, thus can be applied to a variety of developmental biology and neurobiology research. Moreover, KSR, which has a high cost, is essential in EB formation in serum-free cultures (SFEB method established by Kiichi Watanabe et al. Nat Neurosci. 2005), thus such a method was not listed in our studies. But we fully agree with your comments on testing other differentiation conditions. In the further study, we will test more conditions and provide more choices for the researchers. We hope our response are satisfactory. Special thanks to you for your valuable comments.

Q2. Retinoic acid treatment induces caudalization during neural differentiation. Using the method generated in this manuscript, which kind of neuron is induced? The authors are suggested to analysis the neuronal subtype specific markers.

A2. Thank you very much for your valuable comments. We have adopted your suggestions and gave detailed description of neuronal subtype using our optimized method in the revised manuscript (Line 445-447). Using RT-PCR, we have tested the forebrain maker forkhead box G1 (FOXG1), forebrain midbrain marker orthodenticle homeobox 2 (OTX2), hindbrain markers engrailed homeobox 2 (EN2) and paired box 2 (PAX2), anterior hindbrain marker gastrulation brain homeobox 2 (GBX2) and spinal cord marker NeuN. Results showed that EN2, PAX2, GBX2 and NeuN were up-regulated notably in our derivatives on D18, indicating the generation of hindbrain and spinal cord progenitors. However, these results were listed in our other unpublished manuscript (under review), and cannot be reused here. To give a more effective guidance for readers, we quoted other published articles in our revised manuscript (Line 445-447). We hope that the revisions are satisfactory.

Minor concerns:

Q3. The neural differentiation method of mouse and human ESCs are much different from each other. As a method article, the "mouse embryonic stem cells" should be clearly indicated in the title.

A3. Thank you for your kind suggestions. We fully agree with your comments and have revise the title in our revised manuscript.

Q4. The date when the immunofluorescence was performed should be shown in Figure 4B.

A4. Thank you for your kind suggestions. We have added the date when the immunofluorescence was performed on the revised Figure 4B. Special thanks to you for your valuable comments.

We hope that these revisions are satisfactory and that the revised version will be acceptable for publication. Finally, we would like to express our great appreciation again to you and reviewers for your kind comments and suggestions for our manuscript.

Thank you and best regards.

Yours sincerely,

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Wuhan, Hubei, 430065, P.R. China

Email: zhaoshasha@wust.edu.cn

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