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Neurogenesis using P19 Embryonal Carcinoma Cells

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TITLE:

Neurogenesis using P19 Embryonal Carcinoma Cells

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KEYWORDS:

neurogenesis, P19 cell line, aggregates, retinoic acid, neurons, differentiation.

SUMMARY:

The P19 mouse embryonic carcinoma cell line (P19 cell line) is widely used for studying the molecular mechanism of neurogenesis with great simplification compared to *in vivo* analysis. Here, we present a protocol for retinoic acid-induced neurogenesis in the P19 cell line.

ABSTRACT:

The P19 cell line derived from a mouse embryo-derived teratocarcinoma has the ability to differentiate into the three germ layers. In the presence of retinoic acid (RA), the suspension cultured P19 cell line is induced to differentiate into neurons. This phenomenon is extensively investigated as a neurogenesis model *in vitro*. Therefore, the P19 cell line is very useful for molecular and cellular studies associated with neurogenesis. However, protocols for neuronal differentiation of P19 cell line described in the literature are very complex. The method developed in this study are simple and will play a part in elucidating the molecular mechanisms in neurodevelopmental abnormalities and neurodegenerative diseases.

INTRODUCTION:

During embryonal development, a single cell layer is transformed into three separate germ layers[1-3]. To increase the research possibilities of phenomena occurring *in vivo*, generation of three-dimensional aggregates (embryonic bodies) have been developed as a convenient model. Cellular aggregates formed in this way can be exposed to various conditions causing cell differentiation, which reflect development of the embryo[4-5]. The P19 murine embryonic carcinoma cell line (P19 cell line) is commonly used as a cellular model for neurogenesis studies *in vitro*[6-8]. The P19 cell line exhibits typical pluripotent stem cell features and can differentiate into neurons in the presence of retinoic acid (RA) during cell aggregation followed by neurite outgrowth under adherent conditions. Moreover, the undifferentiated P19 cell line is also capable of forming muscle- and cardiomyocyte-like cells under the influence of dimethyl sulfoxide (DMSO)[9-12].

Many methods[13-16] have been reported for neuronal differentiation, but the methodology is sometimes complicated and not easy to grasp by only reading the descriptions. For example, protocols sometimes require a combination of Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with a mixture of calf serum (CS) and fetal bovine serum (FBS)[13]. Moreover, media used for neuronal development are often composed of Neurobasal and B27 supplements[13-16]. As such, existing methods contain complexity in their preparation and our goal here is to simplify the protocols. In this study, we demonstrated that DMEM with FBS can be utilized for maintaining the P19 cell line (DMEM + 10% FBS) as well as for neuronal development (DMEM + 5% FBS + RA). This simplified method for neurogenesis using the P19 cell line allows us to study the molecular mechanism of how neurons are developed. Moreover, research on neurodegenerative diseases such as Alzheimer's disease is also conducted using P19 cell line[17-18], and we believe that the method developed in this study will play a part in elucidating the molecular mechanisms in neurodevelopmental abnormalities and neurodegenerative diseases.

PROTOCOL:

1. Culture Maintenance

1.1. Culture the P19 cell line in Maintenance Medium (Dulbecco's modified Eagle's medium with 4,500 mg/L of glucose supplemented with 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin). Incubate at 37 °C and 5% CO_2.

2. Sub-culturing Cells

2.1. When cells reach approximately 80% confluence, remove the spent medium from the cell culture flasks (surface area 25 cm^2).

2.2. Wash the cells with 2 mL of phosphate buffered saline (PBS) free of calcium and magnesium.

2.3. Add 1 mL of 0.25% trypsin-EDTA (ethylenediaminetetraacetic acid) onto the cell monolayer.

2.4. Put the flask in the CO_2 incubator (37 °C and 5% CO_2) for 5 min.

2.5. Assess the cell attachment to the flask surface. Ensure that all of the cells are detached and floating in the medium.

2.6. Add 9 mL of Maintenance Medium to inactivate the enzymatic activity of trypsin.

2.7. Resuspend the cells in Maintenance Medium.

2.8. Transfer cells to a 15 mL tube and centrifuge for 5 min at 200 x g and room temperature (RT).

2.9. Discard the supernatant and add 10 mL of fresh Maintenance Medium into the 15 mL tube.

2.10. Use the cell suspension to determine the cell number using a cell counter according to manufacturer's instructions.

2.11. Seed cells at 2 x 10^4 cells/cm^2 in a new 25 cm^2 flask.

2.12. Add the Maintenance Medium up to 10 mL.

2.13. Put the flask with cells in the CO_2 incubator (37 °C and 5% CO_2) for 2-3 days.

3. Trypsin Digestion

3.1. Aspirate Maintenance Medium from the cell flask. Wash the cells once with 5 mL of calcium and magnesium-free PBS.

3.2. Add 1 mL of 0.25% trypsin-EDTA.

3.3. Put the flask with cells into the CO_2 incubator at 37 °C for 2-3 min.

3.4. Use 1 mL pipette to dissociate the cells by pipetting cells ten times.

3.5. Neutralize trypsin by adding 8 mL of Differentiation Medium (Dulbecco's modified Eagle's medium with high glucose level supplemented with 5% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin) without RA to the cells.

3.6. Transfer cells to a 15 mL tube and centrifuge for 5 min at 200 x g and RT.

3.7. Discard the supernatant and add 1 mL of Differentiation Medium without retinoic acid (RA). Resuspend the cell pellet.

3.8. Use the cell suspension to determine the cell number using a cell counter according to manufacturer's instruction.

4. Aggregate Generation

4.1. Add 5 µL of RA (1 mM stock dissolved in 99.8% ethanol, stored at -20 °C) to the 10 mL of Differentiation Medium and mix well (final concentration of 0.5 µM RA).

NOTE: RA is light sensitive and the low concentration of EtOH does not affect cell differentiation[19-21].

4.2. Add 10 mL of Differentiation Medium (with RA) to the 100 mm non-treated culture dish (dedicated to suspension culture).

4.3. Seed the 1 x 10^6 cells in the 100 mm dish (Dish surface area 56.5 cm^2).

4.4. Put the flask with cells into the incubator at 37 °C and 5% CO_2 for 2 days in order to promote aggregates formation.

4.5. After 2 days, exchange the Differentiation Medium. Aspirate medium containing aggregates using a 10 mL pipette and transfer to a 15 mL tube at RT.

4.6. Allow the aggregates to settle by gravity for 1.5 min at RT.

4.7. Discard the supernatant.

4.8. Add a fresh 10 mL of Differentiation Medium with 0.5 µM RA using a 10 mL serological pipette.

CAUTION: Do not pipette the cell aggregates up and down.

4.9. Seed the aggregates into new 100 mm non-treated culture dish (dedicated to suspension culture).

4.10. Place the plate in the incubator (at 37 °C and 5% CO_2) for 2 days.

5. Aggregates Dissociation

5.1. Aspirate the cell aggregates using a 10 mL pipette.

5.2. Transfer the aggregates to a 15 mL tube. Allow the cell aggregates to settle by gravity for 1.5 min.

5.3. Remove the supernatant.

5.4. Wash the aggregates with DMEM alone (serum- and antibiotic- free).

5.5. Allow the cell aggregates to settle by gravity sedimentation for 1.5 minutes at RT.

5.6. Aspirate the supernatant and add 2 mL of trypsin-EDTA (0.25%).

5.7. Place the cell aggregates into a water bath (37 °C) for 10 min. Agitate the aggregates gently every 2 min by tapping with a hand.

5.8. Stop the trypsinization process by adding 4 mL of Maintenance Medium.

5.9. Pipette aggregates up and down 20 times using 1 mL pipette.

5.10. Centrifuge cells for 5 minutes at 200 x g and RT.

5.11. Remove the supernatant and resuspend the cell pellet in 5 mL of Maintenance Medium.

5.12. Determine the cell number with a cell counter.

6. Plating Cells

6.1. Add 3 mL per well of Maintenance Medium to a 6-well plate.

6.2. Seed cells in the 6-well culture plate at a density of 0.5 x 10^6/well.

6.3. Incubate at 37 °C with 5% CO_2 concentration.

6.4. Seed the cells on cover glass in 6 well culture plate and perform immunostaining with anti-MAP2 antibody (20% confluence). Use 6-well plate to isolate RNA and perform RT-PCR for *Map2*, *NeuN*, *Oct4*, *Nanog*, and *Gapdh* (20% confluence).

REPRESENTATIVE RESULTS

The simplified scheme of protocol for neurogenesis induction in P19 cell line is presented in **Figure 1**. In order to define the character of the P19 cell line in an undifferentiated state and during neurogenesis, the RT-PCR (reverse transcription-polymerase chain reaction) method was used. The undifferentiated P19 cell line expressed the pluripotency genes such as *organic cation/carnitine transporter4 (Oct4)* and *Nanog homeobox (Nanog).* Neurogenesis induced by cells aggregation in suspension culture in the presence of RA led to a rapid decrease of *Oct4* and *Nanog* expression. In contrary, expression of neuron markers: *microtubule-associated protein 2* (*Map2)*, *NeuN* (also known as *RNA binding protein, fox-1 homolog 3 (Rbfox3))* increased after triggered neurogenesis (**Figure 2**)[6,14,15,22]. The primers used for each gene are indicated along with nucleotide sequences and the size of the product in **Table 1.** A microscopic image of the undifferentiated P19 cell line presented a round-shaped morphology (**Figure 3A**). After induction of neurogenesis, the neuronal structure of the cells was clearly visible 4 days after plating (**Figure 3B**). Additionally, **Figure 4** represents the fluorescence image of MAP2 expression in the differentiated P19 cell line (4 days after plating cells)[23].

Figure 1. Protocol schematic for induction of neurogenesis in P19 embryonal carcinoma cells. Neurogenesis is induced by culturing the P19 cell line in a 100 mm non-treated culture dish with 5% of FBS and 0.5 µM RA. After 4 days, the cell aggregates are dissociated with trypsin and seeded on adherent cell culture plate for following next 4 days.

Figure 2. Changes of gene expression in P19 cell line. The band graph represents gene expression for undifferentiated P19 cell line (*Oct4, Nanog*) and during neurogenesis (*Map2, NeuN*). *Glyceraldehyde-3-phosphate dehydrogenase (Gapdh)* was used as the reference gene. Samples are loaded in the agarose gel (1.5%) in double replications. Abbreviations: Undifferentiated represents the undifferentiated P19 cell line without RA treatment; Day 1-4 represents subsequent days after cell plating- following 4 days after RA treatment and cell aggregation stage.

Figure 3. Representative images of analysis of P19 cell line. (**A**) Light microscopic images of undifferentiated P19 cell line. (**B**) Light microscopic images of P19 cell line after 4 days of neurogenesis- following 4 days after RA treatment and cell aggregation stage. Scale bar = 100 µm.

Figure 4. Representative immunofluorescence image of differentiated P19 cell line. Merged immunofluorescence image of P19 cell line stained with anti-MAP2 and DAPI at 4 days after plating. Scale bar = 100 µm.

Table 1: Primers used for RT-PCR.

DISCUSSION:

Here, we describe a simple protocol for neurogenesis using the P19 cell line. Although many reports have been published in this regard, a detailed methodology for neurogenesis induction using P19 cell line remains unclear. Moreover, we utilized a simple high glucose DMEM medium with 10% FBS for the entire experiment. This allowed us to perform the neurogenic experiment in a user-friendly manner and expand the usage of this method for the future.

The most critical points within this protocol are the RA concentration as well as the generation of cell aggregates in the suspension culture. The stimulation of neurogenesis in the P19 cell line can be carried out without the formation of aggregates, but the number of neuronal cells produced will be reduced by two-thirds in the cell culture[22]. Monzo *et al.* have shown neurogenesis induction in P19 cell line by culturing them in monolayer[15]. Although their method is quite convenient as we can eliminate suspension culture process, further studies are required to compare their method with other well-described methods. The RA concentration of 0.5 µM in the medium produced a high number of cell aggregates as well as neurons after plating as compared to 1 µM of RA. It is also important to note that we could not observe an efficient neurogenesis when most of the aggregates are attached to the bottom of the suspension culture dish during RA treatment. The optimal number of the P19 cell line to be used at the beginning of the procedure is 1 x 10^6 for every 10 mL of Differentiation Medium. During the induction of neurogenesis, the P19 cell line forms varying sized aggregates and even single cells are found in the culture. To overcome this problem, we collected the cell aggregates after 1.5 minutes of free fall in a 15 mL tube. We found that this approach allows the exclusion of contamination of single cells. It is also recommended to perform neuronal enrichment with the cell culture using anti-mitotic drugs (*e.g.*, cytosine arabinoside) for long term culture to inhibit extensive proliferation of glial cells[24].

Neurons derived from the P19 cell line express ionotropic glutamate receptors of both N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainite (KA) types[25], as well as functional γ-aminobutyric acid (GABA) receptors[26]. Therefore, the P19 cell line is widely used in the studies on molecular mechanisms governing neuronal differentiation[27-29]. More importantly, the tumor development was not observed after cell transplantation[30-31].

To this end, research on neurodegenerative diseases such as Alzheimer's disease[17,18] is also conducted using P19 cell line, and we believe that the method developed in this study will thus play a part in elucidating the molecular mechanisms in neurodevelopmental abnormalities and neurodegenerative diseases.

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DISCLOSURES

The authors have nothing to disclose.

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Figure 1

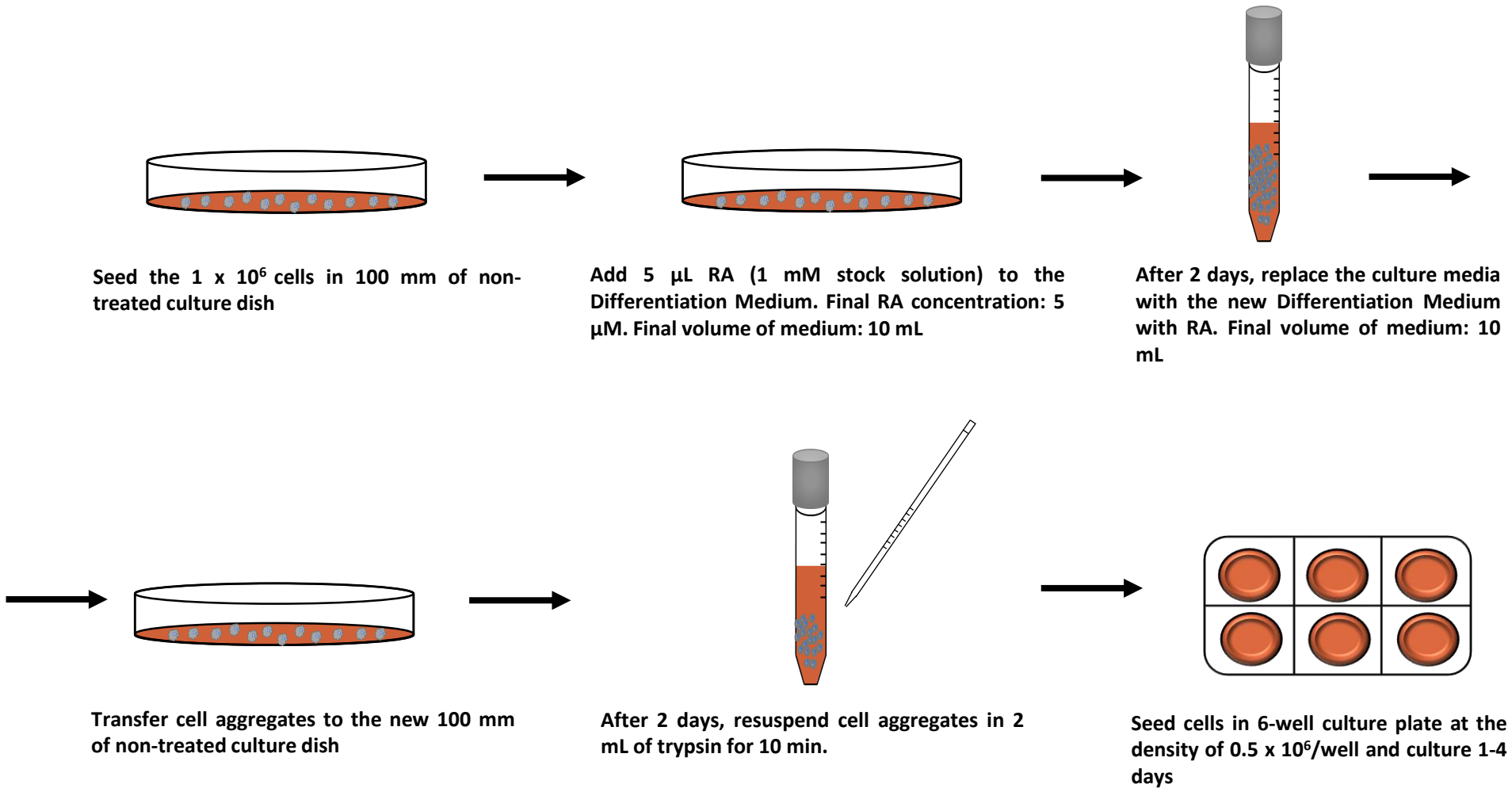


Figure 2

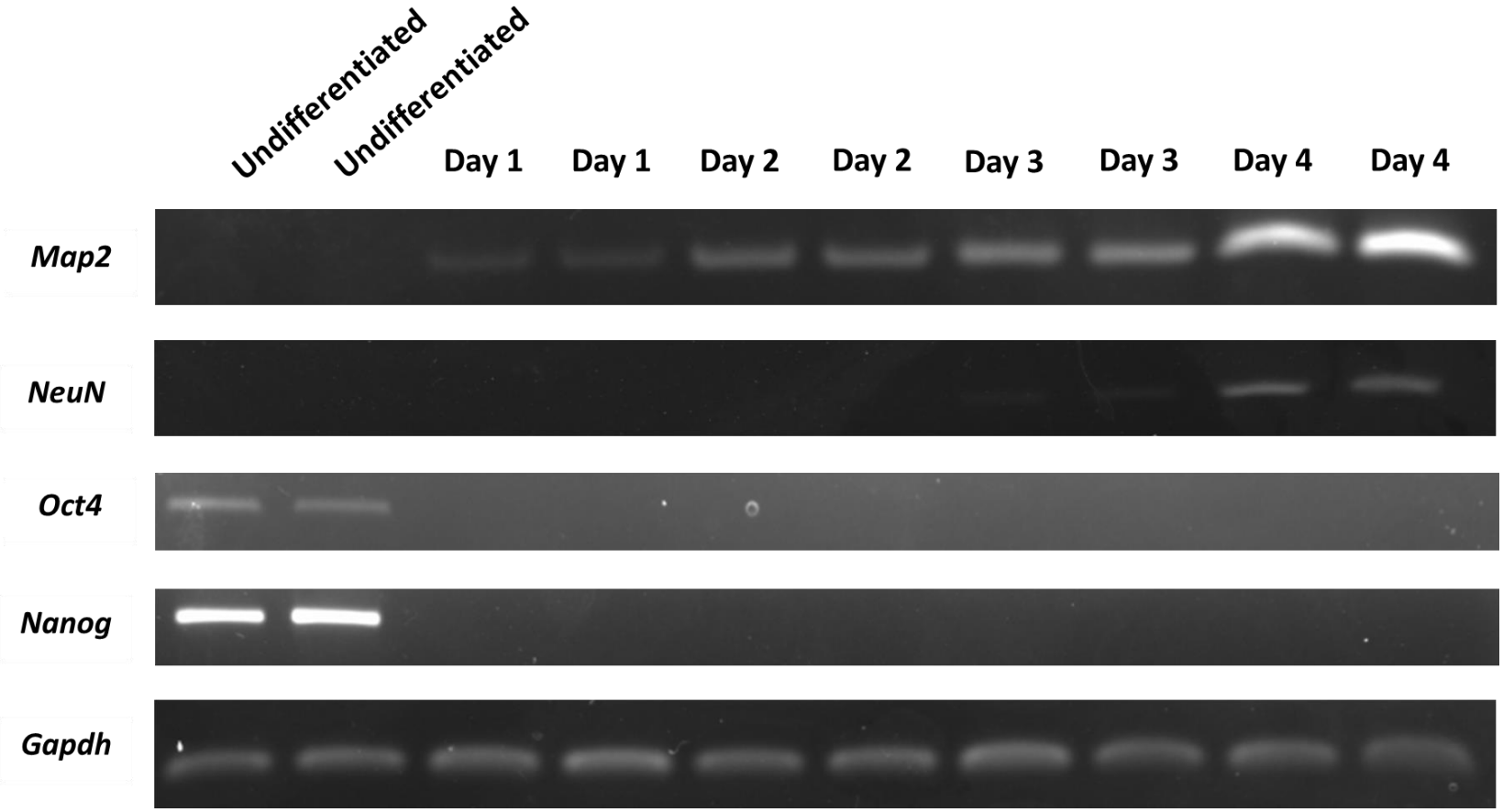
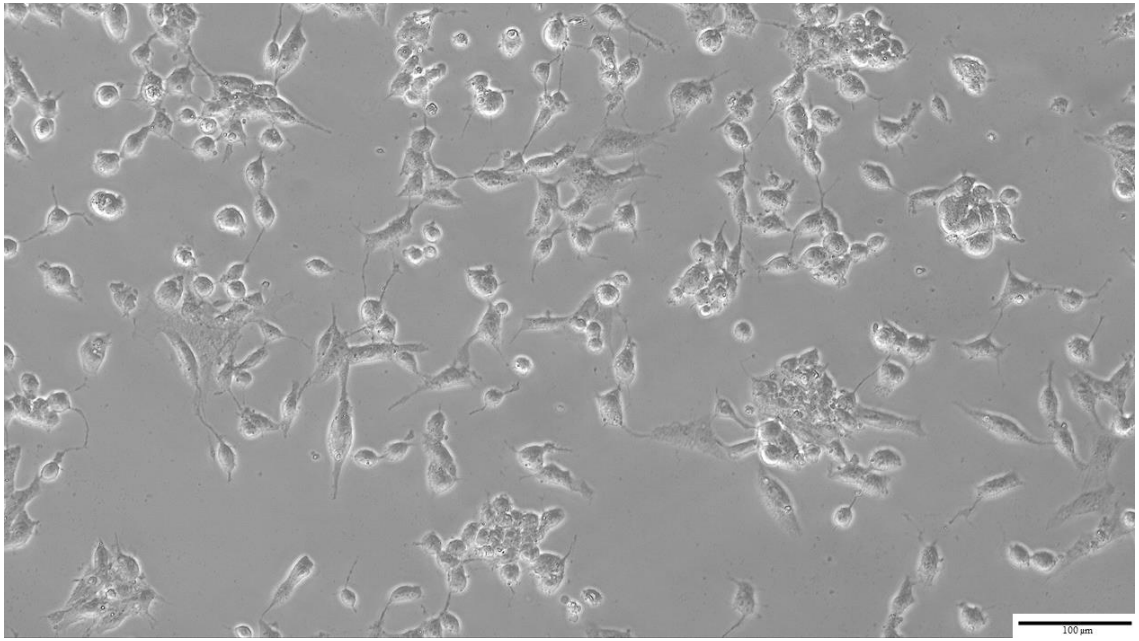


Figure 3

A

Undifferentiated



B

Day 4

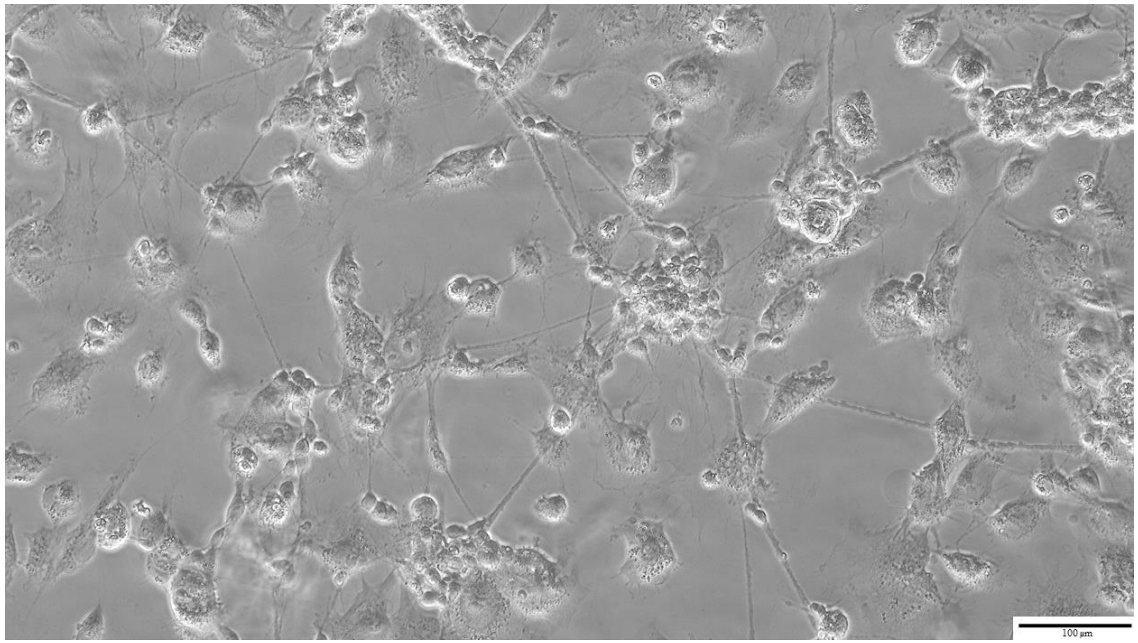
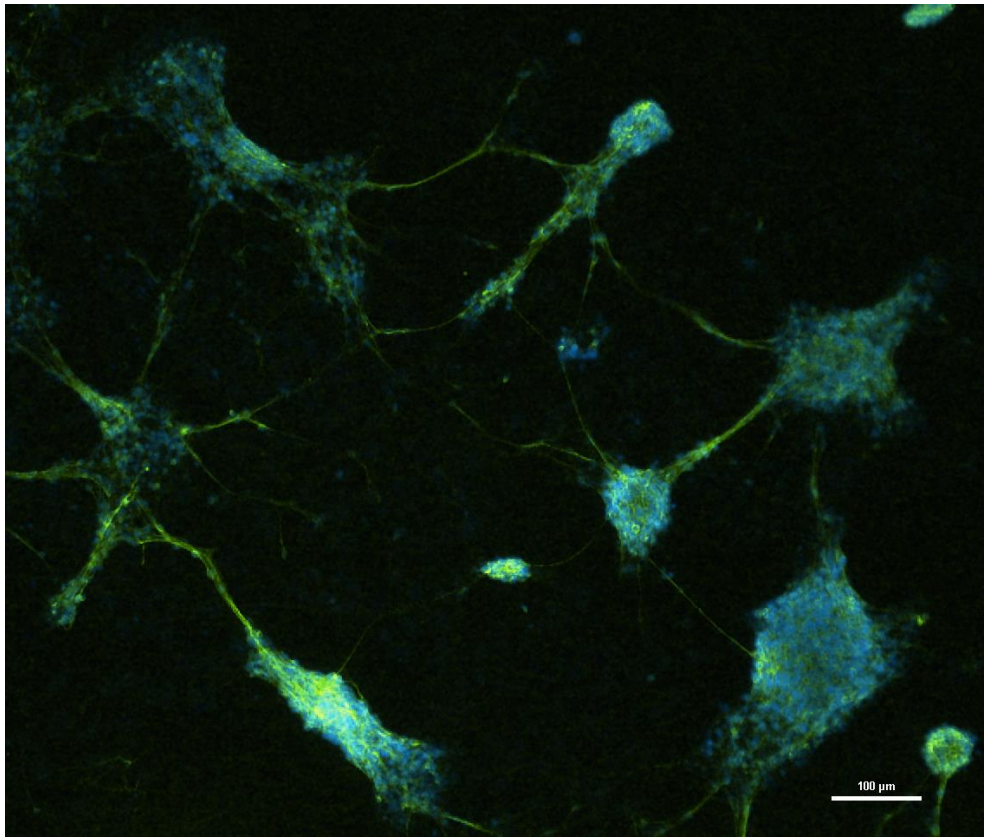


Figure 4



Primer	Primer sequence	Product size (bp)
Gapdh	F: TGACCTCAACTACATGGTCTACA	85
	R: CTTCCCATTCTCGGCCTTG	
Map2	F: GCTGAGATCATCACACAGTC	211
	R: TCCTGCCAAGAGCTCATGCC	
Oct4	F: GGCGTTCTCTTTGGAAAGGTGTTC	313
	R: CTCGAACCACATCCTTCTCT	
NeuN	F: GGCAAATGTTCGGGCAATTCG	160
	R: TCAATTTTCCGTCCCTCTACGAT	
Nanog	F: AAAGGATGAAGTGCAAGCGGTGG	520
	R: CTGGCTTTGCCCTGACTTTAAGC	

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
DMEM high glucose (4.5 g/l) with L-glutamine	Lonza	BE12-604Q	
Trypsin 0.25% - EDTA in HBSS, without Ca2+, Mg2+,with Phenol Red	biosera	LM-T1720/500	
Fetal Bovine Serum (FBS)	EURx	E5050-03	
Penicillin/Streptomycin 10K/10K	Lonza	DE17-602E	
Ethanol 99.8%	Chempur	CHEM*61396 4202	
Phosphate Buffered Saline (PBS), 1x concentrated without Ca2+, Mg2+	Lonza	BE17- 517Q	
Retinoic acid	Sigma-Aldrich	R2625-50MG	dissolved in 99.8% ethanol; store in -20 °C up to 6 months

MAP2 antibody	Thermo Fisher Scientific	PA517646	Dilution 1:100
Secondary Antibody (Alexa Fluor 488)	Thermo Fisher Scientific	A11034	Dilution 1:500
DAPI (4′,6-Diamidine-2′-phenylindole dihydrochloride)	Sigma-Aldrich	10236276001	Working concentration: 1 µg/mL
cDNA synthesis kit	EURx	E0801-02	
PCR reaction kit	EURx	E0411-03	
Agarose	Sigma-Aldrich	A9539	
Skim milk	Sigma-Aldrich	1153630500	
Triton-X 100	Sigma-Aldrich	T8787-100ML	

TBE Buffer	Thermo Fisher Scientific	B52	
6X DNA Loading Dye	EURx	E0260-01	

Cell Culture Plastics			
Cell culture flasks, surface area 25 cm2	Sigma-Aldrich	CLS430639-200EA	
100 mm dish dedicated for suspension culture	Corning	C351029	
6-well plate	Corning	CLS3516	
15 mL centrifuge tubes	Sigma-Aldrich	CLS430791-500EA	
1 mL Serological Pipettes	profilab	515.01	
5 mL Serological Pipettes	profilab	515.05	
10 mL Serological Pipettes	profilab	515.10	

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Signature:	
Date:	19 Oct 2018

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Oct20, 2018

Dear Dr. Vineeta Bajaj

Please find the attached revised manuscript entitled “Neurogenesis using P19 Embryonal Carcinoma Cells”, which we would like you to consider for publishing in Journal of Visualized Experiments. We have substantially revised the manuscript to address all concerns and suggestions of the editor and the three reviewers and submitted the revised version for your kind perusal and consideration. We hope that the current version will now meet publication requirements in Journal of Visualized Experiments. We have confirmed that the revised manuscript fulfills the criteria suggested by you.

We thank you for extending the deadline for resubmission and should you have any queries, please feel free to ask me.

Best regards,

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Editorial comments:
Changes to be made by the Author(s):
1. 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.

Thank you for your constructive comment. We have checked the manuscript again.

2. Figure 2: Please include a space between the number and the units of the scale bar.

Thank you for your constructive comment. We have modified Figure 2 as suggested.

3. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

Thank you for your constructive comment. We have modified the summary as suggested.

4. Please revise the Introduction to include all of the following:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application

Thank you for your constructive comment. We have newly added the information regarding e) Information to help readers to determine whether the method is appropriate for their application. We have checked the Introduction again and confirmed that our manuscript fulfills the criteria.

5. Please define all abbreviations before use (PBS, etc.).

Thank you for your constructive comment. We have defined all the abbreviations.

6. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Thank you for your constructive comment. We have modified the descriptions as suggested.

7. 1.2: Please describe how cell passage is done.

Thank you for your constructive comment. We have described the process in the revised manuscript as suggested.

8. 2.3: Please specify the incubation temperature.

Thank you for your constructive comment. We have modified the description as suggested.

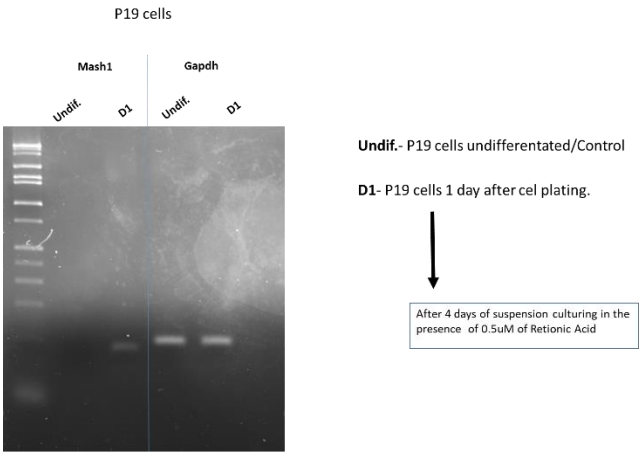
9. References: Please do not abbreviate journal titles.

Thank you for your constructive comment. We have modified the journal titles as suggested.

Reviewers' comments:

Reviewer #1:
JOVE58225: A simple method for inducing neurogenesis in P19 embryonal carcinoma cells
This manuscript by Pawel Leszczynski et al, focuses on the description of a protocol for inducing neurogenesis from P19 embryonal carcinoma cells.

protocol concerns:
*In our experience, culturing P19 cells in 10% FCS might provide a fraction of neuronal autodifferentiated cells due to the presence of traces of retinoid analogs, reason why 5%FCS + 5%FCS delipidated are used for decreasing such autodifferentiation potential. Do the authors observed potential autodifferentiation events during their culture maintenance? In Figure 2 we can observe some filamentous-like structures which on our experience could suggest the presence of an autodifferentiation potential. Furthermore in Figure 1 authors evaluates the level of expression of a certain number of markers to illustrate the gain in neuronal markers as well as the loss of stemness, or early responding markers during neurogenesis (e.g. Ascl1, Tal2 as illustrated by Kobayashi et al; SciRep. 2014) are missing, mainly to evaluate the presence of potential autodifferentiation.



Thank you for your constructive comment. During P19 cell culture maintenance we did not observe autodifferentiation of P19 cells. We also verified the expression of Mash1 (Ascl1) in undifferentiated P19 cells (Undiff.) and D1 (1 day after cell plating-5 days after induction of neurogenesis during cell aggregation with the retinoic acid). Since we did not observe the expression of Mash1 in undifferentiated P19 cells whereas the expression was found in D1, it is concluded that P19 cells are not autodifferentiated in our culture system.

*While the protocol seems to generate neurons, the performance over other methods is not evaluated. Notably there are two major aspects that is not discussed/evaluated in this protocol: (i) the performance of cell aggregates versus the monolayer strategy described by Monzo et al (J. Neurosci Methods; 2012); (ii) the use of RA concentrations higher than previously described. This last point is of major relevance in the context of other strategies, namely those aiming to avoid the use of retinoic acid during neuro-ectodermal inuction for avoiding induction of Hox-related patterning. Overall, while I do agree that the procedure described on this manuscript allows to induce neurogenesis, none of the provided figures allows to judge about the yield of the procedure (fraction of neuronal cells induced after 2 +4 days of culture), neither to evaluate its efficacy relative to other well described methods.

Thank you for your constructive comment. From what we have tested regarding the points you raised, we think making aggregates in the suspension culture is one of the most important points in our protocol. On the other hand, Monzo et al. has clearly demonstrated that neurogenesis can be introduced by the monolayer strategy. We appreciate their technique to introduce neurogenesis. However, their technique requires a matrigel coating plate for their neuronal differentiation. Moreover, it takes 14Days after 1uM RA treatment to observe neuronal-like cells in large numbers. On the other hand, our method allows us to observe similar cell morphology 8Days after 0.5uM RA treatment. Therefore, it is concluded that our method can still be useful in the field of neuroscience. More importantly, we use similar concentration of RA as previously reported studies including Monzo et al. Nevertheless, further studies are required for an optimization of RA concentration to avoid its side effect, and for comparison of our protocol and other methods using functional assays . To clarify this point, we have added the following sentence in the Discussion. "Monzo et al. have clearly demonstrated that neurogenesis is introduced by the monolayer culture method using P19 cells. Although their method is quite convenient as we can eliminate suspension culture process, further studies are required to compare their method with other well-described methods.". Thank you very much for your very

important suggestions.

Reviewer #2:
Manuscript Summary:
The manuscript entitled "A simple method for inducing neurogenesis in P19 embryonal carcinoma cells" presents a simple, easy and cheap method to differentiate P19 cell line into neurons.

Major Concerns:
Some improvement is necessary to clarify the described methods. Please consider the following comments and suggestions:
1) In the Abstract and Introduction please change P19 cells for P19 cell line, to clearly state it is a cell line;

Thank you for your constructive comment. The description of P19 cells in the manuscript was changed from "P19 cells" to "P19 cell line (P19 cells)" and the same modifications were performed wherever appropriate.

2) A scheme containing timings for each procedure/step should be included for easily and rapidly assessing the whole procedure

Thank you for constructive comment. We have newly added a scheme containing timings for each procedure/step

3) Is it necessary to have a final step of neuronal enrichment with cell culture using anti-mitotic drugs? Discuss it

Thank you for your constructive comment. During first days after cell plating mostly indicated cells display neuronal markers. Our protocol was designed to finish cell culture after 4 days of cell culture where number on neuron-like cells is high. We agree that anti-mitotic agent should be added to cell culture but only in situation when researcher is aiming to prolong his studies. Therefore, we have added the following sentence in the Discussion.

" It is also recommended to perform neuronal enrichment with cell culture using anti-mitotic drugs (e.g. Cytosine arabinoside) for long term culture to inhibit extensive proliferation of glial cells."

4) Which is the final neuronal production yield? It should be included an average number of neuronal production whenever starting the process with 1x106 cells, for instance. Is the final yield an advantage?

We assume that the final production yield can be evaluated by Map2 staining (neuronal marker) and therefore we have newly added Figure 4 to clarify this point. Our staining result shows that almost all the cells possess neuronal character.

5) In figure 1 clarify whether the days are counted during aggregate-based differentiation process

Thank you for your constructive comment. In the revised version of the manuscript we added the missing information. In our protocol we started counting days from the time P19 cell were replated on culture plates (after 4 days of RA treatment). We have added the following sentences to clarify this point. "Neurogenesis is induced by culturing P19 cells in 100 mm of non-treated culture dish with 5% of FBS and 0.5 µM RA (The cells form aggregates). After 4 days, the cell aggregates are dissociated with trypsin and seeded on adherent cell culture plate for following next 4 days."

6) In Figure 4B: does "day4" mean 4 days after plating being 8 days after starting neuronal differentiation process? Clearly state it

Thank you for your constructive comment. In the revised version of manuscript we clarified that Day 4 represents 8 days after the induction of neurogenesis (RA treatment).

Minor Concerns:
General comments about the protocol:
1.2 - about 90% of confluency?

Thank you for your constructive comment. We have added the description (70-90% of confluency) in the manuscript.

3.1 - please state temperature of RA storage

Thank you for your constructive comment. We stored RA at -20 and we have added the description in the manuscript.

3.2 - Clearly mention that differentiation medium is with RA. Better define what is dish dedicated to suspension culture. Please define the surface area for this dish.

Thank you for your constructive comment. We have clearly mentioned that differentiation medium is with RA and described catalog number of suspension culture dish in the materials section.

3.3 it is mentioned dish and in 3.4 it is mention flask; please maintain the same name for avoiding misunderstandings

Thank you for your constructive comment. We have changed from "flask" to "plate" in 3.4 of the manuscript.

5.1 - 3 mL per well - clearly state

Thank you for your constructive comment. We have added "3 ml per well" to 5.1 of the manuscript.

Reviewer #3:

The manuscript entitled: "A simple method for inducing neurogénesis in P19 embryonal carcinoma cells." Introduce a simple method to differentiate the P19 cells into neuronal cells, employing accessible reagents and describing the process step by step to successfully fulfill the protocol. I believe that the protocol described will benefit the scientific community, however some steps should be further clarified to make it more clear and accessible for researchers not used to work with P19 cells.

Minor and Major Concerns:

-In the introduction, authors state that "DMEM with 10% FBS for maintaining P19 cells as well as for neuronal development can be utilized", however in the protocol it is described Differentiation medium, (DMEM with 5% FBS) and subsequent Retinoic acid is required for the differentiation into neurons. This is somehow confusing and should be clarified.

Thank you for your constructive comments and we are sorry that our description was confusing. We meant that we only use FBS and RA for our neurogenesis which is relatively convenient compared with the method in which we should use FCS and FBS. Therefore, we have changed the description as follows: "In this study, we demonstrated that DMEM with FBS can be utilized for maintaining P19 cells (DMEM+10%FBS) as well as for neuronal development (DMEM+5%FBS+RA)"

PROTOCOL:

Step 1.2- How is the cell passage performed? In which kind of plate are the cells maintained?, flask? Which size? Is there any coating needed?

Thank you for your constructive comments and we have described catalog number of our culture flask in the material section. We have also added the detailed method how we maintain the cells in sections 1-2.

Step 2.9- and 4.12- It is possible to add an alternative method for cell counting?

Thank you for this comment. We have changed this description to "cell counter" instead of hemocytometer.

Step 3.3 and 5.2- It is any specific coating required for seeding the cells? Are the aggregates free-floating or they are attached to the plate surface? Please specify

Thank you for this comment. We do not need specific coating for plating the cells and the aggregates are floating in the suspension culture plate.

Step 3.6 and 4.5- Is this step performed at RT or 37ºC?

Thank you for this comment. We have added the missing information about the temperature. We have added "room temperature" in our revised manuscript.

Step 5.3- After this step it is to be assumed that the cells are already neurons ? Could the authors specify this on the protocol? If so, should step 5.3 correspond then to the Day 1 depicted in figure 1?

Thank you for this comment. It normally takes 4-6days after plating the cells to a 6 well plate and we have clarified this in the result section.

Figure 2B would strongly benefit with an additional picture of an immunocytochemistry staining with neuronal markers, to support the statements of neuronal differentiation and to discard a potential heterogeneity in the cell culture (comprising both differentiated and undifferentiated cells)

Thank you for this comment. We have performed an immunofluorescent staining for Map2 (neuronal cell marker)(shown in Figure 4).