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A PROTOCOL FOR ISOLATION OF FETAL NEURAL PROGENITOR CELLS FROM MOUSE HIPPOCAMPUS

ABSTRACT

Culture of neural stem/progenitor cells are widely used to study the characteristics of these cells under controlled conditions *in vitro* as well as to study the cellular and molecular mechanisms of CNS diseases and develop strategies for their treatment.

This paper provides a detailed protocol for isolation of fetal (E17-18) neural progenitor cells (*NPCs*) of mouse hippocampus. The technique is based on the use of centrifugation of hippocampal cells suspension in Percoll density gradient to obtain purified NPCs fractions. The cells are cultured in serum-free medium in a monolayer, which creates conditions for more equitable access of *FGF-2* to the cells. This method provides a homogeneous population of undifferentiated progenitors from fetal mouse hippocampus.

KEYWORDS: neural progenitors; neural cell culture; hippocampus

Discovery of neural stem/progenitor cells is one of the greatest achievements in the field of neuroscience. Recently, more and more information is being confirmed about ongoing neurogenesis in certain areas of the mammalian brain, which is provided by a pool of stem cells [1, 2]. A significant step in the study of neural progenitor cells was their isolation and cultivation. NPCs culture is a powerful tool for the dissection of molecular and cellular mechanisms in the field of neuroscience.

An important NPCs feature is the ability to maintain a proliferative activity *in vitro*, allowing to increase their numbers in culture. Although much attention is paid to the potential use of NPCs in cell therapy, neural progenitors culturing may also be important in study of neurogenesis mechanisms under control conditions *in vitro* [3].

Neural progenitor cells were initially cultured as free-floating globular colonies (neurospheres) in serum-free medium with various growth factors such as epidermal growth factor (EGF) [4]. Neurospheres cloning made an instrument for setting of stemness properties in certain populations of primary neural progenitor cells. This approach has shown that some brain regions of the developing and mature mammalian brain, including humans, contain pools of neural stem cells [5-7]. Later, NPCs were cultivated in monolayer cultures using fibroblast growth factor type 2 *FGF-2* [8, 9].

Monolayer (adhesive) culture of neural progenitors has several advantages compared to neurospheres. This culture is a homogeneous population of undifferentiated progenitor cells [10]. Cells in adhesive culture can be easily monitored and assessed by morphology and behavior of each cell. Therefore NPCs monolayer is a potential source of cells for transplantation and useful model for a study of cell migration and differentiation.

Earlier protocols have been developed for cultivation in monolayer of neural progenitors from the subventricular zone of rat's lateral ventricles [9]. A relevant objective is to obtain adhesion culture of neural progenitor cells from fetal mouse hippocampus.

This study presents a protocol developed to isolate pure fraction of neural progenitor cells from fetal (17-18 dpc embryo – E17-18) mouse hippocampus and culture them in a monolayer in serum-free medium with FGF-2.

MATERIALS AND METHODS

All animal experiments were performed in compliance with the article 26 of the Law of Ukraine «On protection of animals from cruelty» (from 21.02.2006.), European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (Strasbourg, 1986), and in compliance with all principles of bioethics and biosafety regulations.

THE NECESSARY MATERIALS AND REAGENTS

№	MATERIALS	COMPANY, CAT. NO
1	Neurobasal medium	Invitrogen, 21103-049
2	B27 supplement	Invitrogen, 17504-044
3	GlutaMAX	Invitrogen, 35050-038
4	Na pyruvate	Sigma, P2256
5	N-Acetyl-L-cysteine	Sigma, A9165
6	PenStrep	Gibco, 15140-122
7	Fibroblast growth factor FGF-2	Sigma, F0291
8	Hanks' balanced salt solution (HBSS)	Sigma, H4641
9	Percoll	GE Health Care BioScience, 17-0891-01
10	Matrigel	BD Biosciences, 354234
11	PBS (phosphate buffered saline)	Gibco, 10010031
12	Cell strainer (40 μm)	BD Falcon, 352340
13	Glass Pasteur pipette	Sigma, S6268
14	35- and 60-mm Petri dishes	Sigma, Z707651, Z707678
15	15- and 50-ml conical tubes	BD Biosciences, 352097, 352070

COMPOSITION OF CULTURE MEDIUM(100 ML)

Neurobasal medium	96.3 ml
B27 supplement	2 ml
GlutaMAX	1 ml
Na pyruvate	100 μl
N-Acetyl-L-cysteine	100 μl
PenStrep	500 μl

PREPARATION OF 22 % PERCOLL SOLUTION, 10 ML

In the 15-ml tube add 7.8 ml of *PBS* and 2.2 ml of 100 % *Percoll*. Mix thoroughly.

PREPARATION OF MATRIGEL SOLUTION AND COATING PETRI DISHES

Prepare 20 ml of Matrigel solution to cover ten 35 mm Petri dishes (2 ml in each). In 50-ml test tube pour 20 ml of *Neurobasal* and add 40 ml of 100% *Matrigel*. Mix thoroughly the resulting solution, fill 35mm Petri dishes with *Matrigel* solution and leave at room temperature for at least an hour (until the cells are collected). Before cell plating, *Petri* dishes are washed twice with *Neurobasal* medium.

PROTOCOL

1. All steps are performed in cold solutions; Petri dishes are placed on ice.
2. Fill three 60-mm Petri dishes with 5 ml (in each) and one 35-mm Petri dish with 1 ml cold *HBSS* and place them on ice.
3. A pregnant mouse at 17-18 dpc is euthanized by cervical dislocation under ether narcotization. Clean the abdominal skin with 70 % ethanol and make a 0.5 cm cross incision in the middle of abdomen. Holding the skin above and below the cut, pull it toward the head and tail to expose access to the abdominal cavity.
4. Make an incision of the peritoneum with other sterile scissors, prepare the uterus with embryos and transfer in 60-mm Petri dish filled with 5 ml of cold *HBSS*.
5. Cut the uterus, get all the embryos and transfer them to the second 60-mm *Petri* dish with 5 ml cold *HBSS*.
6. While holding embryo's neck with forceps, insert the end of curved forceps just above the nose, push it under the skull, and slide it to the back of the head, cutting the skull and skin. Pull the skin along with a skull with the same tweezers and shove the forceps branch carefully under the brain, bring them together and lift with the brain. Transfer the brain to the third 60-mm Petri dish filled with 5 ml cold *HBSS*.
7. Repeat step 6 for all embryos.
8. Cut the brain in half with a scalpel by making a sagittal incision along the midline. Under stereomicroscope prepare the hippocampus of each hemisphere and transfer it to 35-mm Petri dish with 1 ml cold *HBSS*.
9. Repeat the step 8 for all the brains.
10. Minced the hippocampus with microscissors in *HBSS*.
11. Pipetting of hippocampus fragments to a homogeneous suspension with three glass Pasteur pipettes of different diameters. It is necessary to dissociate the tissue slowly and carefully to minimize cell damage.
12. Pass the prepared cell suspension through a nylon cell strainer with a pore size of 40 microns to 15-ml centrifuge tube.
13. Add *HBSS* to the tube up to 10 ml.
14. Centrifuge the cell suspension in *HBSS* at 240 g for 10 min.
15. Aspirate the supernatant and add 1 ml *PBS*. Resuspend cell pellet carefully.
16. Add 10 ml 22 % Percoll solution in a 15 ml tube and centrifuge at 540 g for 10 min.
17. Carefully layer 1 ml of cell suspension on Percoll.
18. Centrifuge the cell suspension at 540 g for 10 min.
19. Collect the supernatant and add 1 ml Neurobasal medium and resuspend cell pellet.
20. Centrifuge the cell suspension at 240 g for 10 min. Repeat the steps 19-20.
21. Collect the supernatant and add 2 ml cultivation medium to the cell pellet, resuspend and count the number of cells.
22. In one 35-mm Petri dish (2 ml medium) $3 \cdot 10^5$ cells are planted. By counting the total number of collected cells in suspension, calculate the required number of Petri dishes and, accordingly, the total volume of culture medium.
23. In the culture medium add 20 ng per 1 ml *FGF-2* and mix thoroughly.
24. Add 2 ml of cell suspension into the resulting culture medium.
25. In washed Petri dishes add a 2 ml cell suspension.
26. Culture the cells in a CO_2-incubator at +37 ^{0}C and 5 % CO_2.

IMMUNOHISTOCHEMICAL ANALYSIS OF NPCs CULTURE

Freshly isolated cells are planted on cover glasses coated with *Matrigel* ($4 \cdot 10^5$ cells per 35-mm culture dish) for the NPCs phenotype analysis. On the third day of cultivation, culture medium was replaced with cold 4 % paraformaldehyde solution (PFA) up to 1 hour for fixation and immunohistochemical staining of cell culture was performed. Cells were incubated with mouse primary monoclonal antibody to Nestin (*Chemicon*, USA) in a solution of 0.1 M *FBS* with 0.5% *BSA* and 0.3% Triton X-100 for 12 hours at +4 ^{0}C. Primary antibody was visualized with secondary anti-mouse antibody conjugated with fluorochrome *AlexaFluor488* (*Invitrogen*, USA). The nuclei of cells were contrasted with fluorescent dye *Hoechst33342* (*Invitrogen*, USA). Stained cell culture was studied with a confocal scanning microscope *FV1000-BX61WI* (*Olympus*, Japan).

On the third day of cultivation hippocampal neural progenitors had mostly round or bipolar shape and thin spines. Immunohistochemical

analysis showed that a small number of cells (up to 4,8 %) expressed markers of mature neurons (beta-tubulin) or astrocytes *GFAP*, which is probably related to contamination of the original cell population after isolation and purification. However, the main number of cells (95.2 %) expressed nestin **(Fig. 1)**, which is typical for hippocampal progenitor cells [11].

Progenitor cells on the Matrigel did not generate neural spheres and not proliferated, but formed colonies and on the 6th day of cultivation allocated evenly over the dish bottom to form a monolayer. Such adhesive culture creates the conditions for more equitable *FGF-2* access to cells, which contributes to the creation of homogeneous population of undifferentiated progenitor cells.

Thus, this protocol provides a purified culture of adhesive fetal neural progenitors from hippocampus of mice.

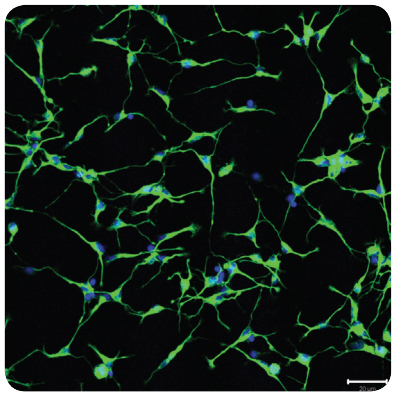

Fig.1. Confocal microscopy of neural progenitors culture. Most cells are positive for nestin (green). Nuclei are contrasted with *Hoechst 33342* (blue). Scale – 20 μm.

REFERENCES

1. *Ma DK, Bonaguidi MA, Ming GL, et al* .Adult neural stem cells in the mammalian central nervous system. Cell Res. 2009; **19(6)**:672-682.
2. *Ming GL, Song H*. Adult neurogenesis in the mammalian brain: significant answers and significant questions. Neuron. 2011; **70(4)**:687–702.
3. *Blakemore WF, Franklin RJ*. Transplantation options for therapeutic central nervous system remyelination. Cell Transplant. 2000; **9(2)**:289–294.
4. *Reynolds BA, Tetzlaff W, Weiss S*. A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. Neurosci. 1992; **12:**4565–4574.
5. *Kukekov VG, Laywell ED, Suslov O, et al*. Multipotent stem/progenitor cells with similar properties arise from two neurogenic regions of adult human brain. Exp. Neurol. 1999; **156(2)**:333-344.
6. *Pagano SF, Impagnatiello F, Girelli M, et al*. Isolation and characterization of neural stem cells from the adult human olfactory bulb. Stem Cells. 2000; **18(4)**: 295-300.
7. *Vescovi AL, Snyder EY*. Establishment and properties of neural stem cell clones: plasticity *in vitro* and *in vivo*. Brain Pathol. 1999; **9(3)**:569-598.
8. *Palmer TD, Ray J, Gage FH*. FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. Mol. Cell Neurosci. 1995; **6**:474–486.
9. *Jenny B, Kanemitsu M, Tsupykov O, et al*. Fibroblast growth factor-2 overexpression in transplanted neural progenitors promotes perivascular cluster formation with a neurogenic potential. Stem Cells. 2009; **7**:1309–1317.
10. *Babu H, Claasen J H, Kannan S, et al*. A protocol for isolation and enriched monolayer cultivation of neural precursor cells from mouse dentate gyrus. Front. Neurosci. 2011; **5**: article 89.
11. *Kempermann G, Jessberger S, Steiner B, et al*. Milestones of neuronal development in the adult hippocampus. Trends Neurosci. 2004; **27**:447–452.

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