Isolation and culture of neural stem cells from murine foetal brain

Nhung Hai Truong, Nhung Thi-Hong Dinh, Dung Minh Le, Linh Thuy Nguyen, Thanh Thai Lam, Ngoc Kim Phan and Phuc Van Pham*

Laboratory of Stem Cell Research and Application, University of Science, Vietnam National University, Ho Chi Minh city, Vietnam

Abstract

There are many mysteries of the nervous system and neurodegenerative diseases. Evidences showed that neural stem cells (NSCs) play a crucial role in recovering nervous system, although this was not sufficient due to lack of cell number. This research was carried out to isolate and culture NSCs from murine foetal brain and our NSCs were cultured as floating and multicellular neurospheres. The neurosphere has been consider as an excellent tool to investigate the differentiation and proliferation of NSCs. Propagation of NSCs *in vitro* was essential for understanding neural cell's fate and supplying a promising source for cell therapy for neurodegenerative disease. Serum-free medium with mitogens, N2 and B27 supplement was appropriate for NSC culture. To characterize candidate cells, we assessed stemness and stem cell markers by sphere formation assay, differentiation assay, and immunocytochemistry. The results showed that NSCs could develop as floating spheres; they expressed stem cell-like characteristics and popular marker of neural stem cells-nestin, and differentiate into nerve cells. This population of cells was a promising source to studying neural stem cells and the treatment of neurodegenerative disease.

Keywords: Neural stem cells; neurospheres; murine foetal brain; neurodegenerative disease; stem cells

Introduction

For a long time, scientists believed that the quantity of nerve cells in the brain is unchangeably indexed. It means that the number of lost nerve cells caused by aging and disease could not be restored during a lifetime. Recently, many researches have proved the presence and roles of neural stem cells (NSCs) in neurogenesis successfully (Fedorrof and Richardson, 2001; Zigova et al., 2002; Doetsch, 2003; Zheng et al., 2007; Doeringering, 2010; Price at al., 2012). In 2002, neuroprotective effects of NSCs were proved in neuronal disorder in mouse models (Ourednik et al., 2002). NSCs are the most primitive form of neural cells. They can be found in their specific niche located at subventricular zone (SVZ) and subgranular zone (SGZ) in the adult brain (Mirescu et al., 2003). NSCs from an adult brain are not as proliferative and easy to propagate than those from the foetal brain, which is

considered a promising source of NSCs (De Filippis et al., 2012). As stem cells from other resources, neural stem cells hold the ability of self-renewal and potential of differentiation into all neural cells including neurons and glias. Hence, once NSCs were isolated and cultivated, they could be promising sources to compensate the disordered areas in neurodegenerative brain (Lindvall et al., 2004).

Because of the small percentage of NSCs *in vivo*, many methods were developed for isolation and culture NSCs *in vitro*. NSCs were first cultured monolayer in poly-L-lysine covered flask. With an adherent condition, these NSCs rapidly differentiated from other neural cell types. The first improvement made to maintain stemness of NSCs is culturing in floating-sphere form, so-called neurospheres. This helps reducing differentiation for a short time. But when NSCs proliferate, they increase the sphere sizes. The bigger the neurospheres grow the easiser they adhere

Corresponding author: Phuc Van Pham, Laboratory of Stem Cell Research and Application, University of Science, Vietnam National University, Ho Chi Minh city, Vietnam

and differentiate. To an advoid sphere adherent, a layer of agarose was employed to improve culturing time. With this method, neurospheres could maintain undifferentiation for 3 months (Louis and Reynolds, 2010).

From primary culture, NSC candidates were used to prove stemness with 3 assays. The first is a sphere-forming assay (Louis and Reynolds, 2010). Candidate cell was cultured in serum-free medium after being isolated from foetal mouse brain according to Reynold and Weiss's protocol and the medium formulation. In this condition, neural stem cells or progeniors can proliferate continuously to form small clusters of cell. These clusters grow in size and form neurospheres. These assays prove the self-renewal ability of NSCs (Louis and Reynolds, 2010). With an advantage of innovative technology, candidate NSCs could be assessed by specific surface markers such as CD133, Sox1, Sox2, Nestin, and CD29 with cell-sorting machines. This assay can reveal how much percentage of NSCs in a candidate cell population. The second golden characteristic of any kinds of stem cell is its differentiation ability (Louis and Reynolds, 2010). These stem cells can be induced to differentiate into many types of neurons in two ways: oriented and un-oriented (Schwindt et al., 2008). NSCs are induced to differentiate unoriented if the medium was modified without its mitogens or adding serum. In another way, with specific growth factor (BNDF, retinoic acid, norepinephrine for neuron induction; RA and LIF for astrocyte induction), specific nerve cell types could be induced (Siebzehnrubl et al., 2011).

Materials and Methods

Neurosphere culture from murine foetal brain

E14 pregnant mice were euthanized and mice foetal taken out from abdominal cavity. Mice foetal were then washed in PBS with 10% of antibiotics (Sigma-Aldrich, St Louis, MO) added twice. Used a sterile blade to take out the brains from the foetal mice and cut into pieces. The brain pieces were digested with 0.025% of trypsin for 10 minutes at room temperature. Trypsin inhibitor (Sigma- Aldrich, St Louis, MO) was used to the stop trypsin activity. Cell suspension was filtered through 70μm cell strainer (BD Bioscience, USA). The cell suspension was centrifuged at 300g for 5 minutes and then removed the supernatant. Cell pellets were washed through PBS and suspended in 4 ml culture medium. Culture medium was the basal medium with high glucose DMEM/F12 1:1 supplemented with 30 μg/ml EGF, 30 μg/ml FGF, 500 IU/ml heparin, B27, 5mg/ml insulin, 1mg/ml transferin, N2 and 1% antibiotic (all bought from Aldrich, St Louis, MO; except B27 from Life Technologies, USA). Culture flasks were coated with 1 mL agarose 1.5%. Cells are cultured in the incubator at 37^0C, 5% CO_2.

Sub-culture neurosphere

Near confluence, neurospheres were washed once with PBS. Neurospheres were dissociated by pipetting with 0.025% of trypsin (Sigma-Aldrich, St Louis, MO) for 5 minutes, and then added trypsin inhibitor to deactivate the trypsin activity. Cell suspension was centrifuged at 300g in 5 minutes. Cell pellets were resuspended in 4 mL culture medium.

Sphere formation assay

Sphere formation assay was used widely to assess cell renewal ability. Neurospheres were dissociated into single cells by trypsinization. Cells were seeded into 24-well plate with concentration at 1000 cells per well and the newly generated neurospheres were recorded.

Differentiation of candidate cells into astrocytes

Differentiation medium was the culture medium with the withdrawal of EGF and bFGF. Cells were seeded into 24-well plate with concentration at 50,000 cells per well. After 3 weeks, differentiated astrocytes were assessed by immunocytochemistry assay. Before staining procedure, cells were fixed by Fixation Buffer (Santa Cruz Biotechnologies, USA) for 30 minutes. Cells were incubated with primary anti-GFAP antibodies (Sigma-Aldrich, St Louis, MO) for 30 minutes and then the secondary rhodamine-conjugated antibodies (Sigma-Aldrich, St Louis, MO) were added. Hoescht 33442 (Sigma-Aldrich, St Louis, MO) was used for nucleus staining. Cells were washed by PBS solution. Expression of GFAP was evaluated by fluorescent inverted microscopy (Carl-Zeiss, Germany).

Immunocytochemistry

Cells were first fixed by incubating with the Fixation Buffer (Santa Cruz Biotechnologis, USA) for 30 minutes. Cell membranes were permeabilized by incubation with Permeabilization Buffer (Santa Cruz Biotechnologies, USA) for 5 minutes. Cells were then incubated with primary anti-nestin antibodies (Sigma- Aldrich, St Louis, MO) for 30 minutes and the secondary FITC-conjugated antibodies (Sigma- Aldrich, St Louis, MO) added later on. Hoescht 33442 was used for nucleus staining. After 30 minutes of incubation, exceeding antibodies were washed away by PBS solution. Expression of nestin was evaluated by inverting fluorescent microscopy (Carl-Zeiss, Germany).

Reverse transcription PCR

The total RNA was extracted from sphere using easy-BLUE Total RNA Extraction Kit (Intron, Korea), according to the manufacturer's protocol. 300ng of total RNA was used to RT-PCR with the primers specific for SOX2, CD133, GFAP, and GAPDH. The sequences of these primers were in the Table 1. The RT-PCR cycling include: 1 cycle in 42°C for 30 minutes, 1 cycle in 94°C

for 5 minutes, 35 cycles in 94°C for 15 seconds, 56°C for 30 seconds, 72°C for 30 seconds, and 1 cycle in 72°C for 10 minutes.

The primary cell suspension contains many cell types: neural stem cells, neural progenitor cells, neural cells, red blood cells, and contaminated cells such as fibroblasts and muscle cells. After 24 hours, the aggregation of cells could be observed. These cell clusters were small and consist of 4 to 8 cells. After 4 days, size of cell cluster increased and some clusters could reach the diameter of 100µm. After 7 days, this cluster became rounder and they became more compact. At this time, they were called neurospheres. Neurospheres increased their volume as long as the inside cells divide. After 14 days, some neurospheres reached diameter of 500 µm (Fig. 1).

When the neurospheres were bigger and attached the surface of flask, they were passaged. Passaging was very important to control cell differentiation. Neurospheres could be dissociated into single cells. A small percentage of these cells could generate new neurospheres. Twenty-four hours after trypsinization, small cell clusters (6-8 cells) could be observed. After 3 days, most of the new spheres reached the diameter of 100 µm (**Fig. 2**). The cells that could not generate new spheres were not stem cells and could be removed by centrifugation.

The expression of nestin
Nestin, an intermediate filament protein, was a popular marker used to identify neural stem cells.

Candidate cells were stained for anti-nestin antibody as both monolayer single cells and neurospheres. Immunocytochemistry result showed that all candidate cells within the sphere were positive for nestin expression. This indicated a typical protein expression of neural stem cells. This result was consistent with the previous reports that virtually all cells in a single neurosphere expressed nestin (**Fig. 3**).

Results

The neurospheres, which diameters were around 100-200 µm were collected and centrifuged for extraction of total RNA. RT-PCR ran with primer specific for three genes: Sox2, CD133 and GFAP. Electrophoresis results showed that cells from neurospheres express Sox2, CD133 and GFAP.

Differentiation into astrocytes
After 3 weeks of differentiation, morphological changes could be clearly seen. From oval shaped with two dendrites, differentiated cells changed to flat polygonal shapes (**Fig. 5**). Moreover, these cells were also positive for GFAP, a specific protein of glial cells (**Fig. 6**). Glial differentiation process happened when mitotic factors were removed from the culture medium. While mitogens were removed, neural stem cells were no longer in proliferation; they switched to differentiation process.
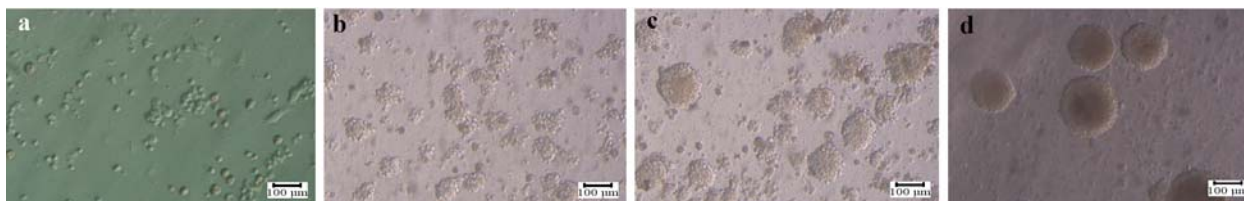

Fig. 1: Primary culture of NSCs from mouse foetal brains. (a) After 12 hours, some cells aggregated together to form cell clusters. The cell clusters contained unpredicted shapes and are not compact. (b) After 2 – 3 days culture, many cell clusters were formed and had various shapes. (c) After 5 – 7 days, the cell clusters were mostly round-shaped and compact. Now they were called neurospheres. (d) After 2 weeks, the neurospheres increased their size; some could reach the diameter of 500 µm.
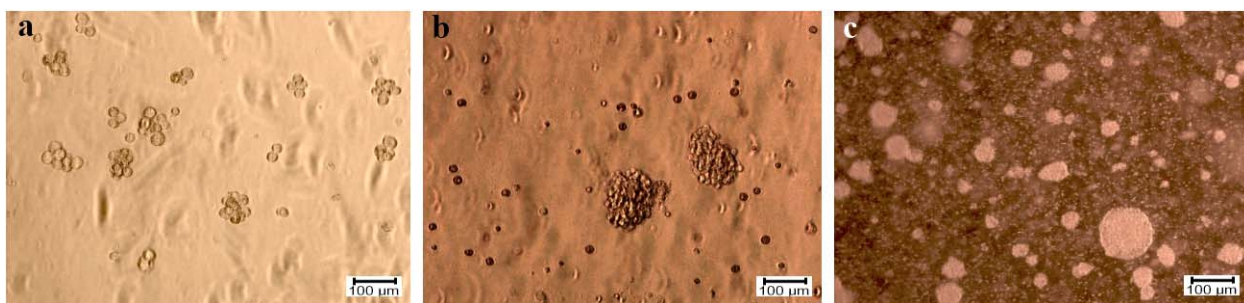

Fig. 2: New neurospheres formation. (a) Cells inside the neurospheres were dissociated into single cells. After 24hrs, new small cells aggregation could be observed. (b, c) After 3 days, the neurospheres were formed. The secondary sphere formation was easier to observe than the primary ones because most of the contaminated cells had been removed
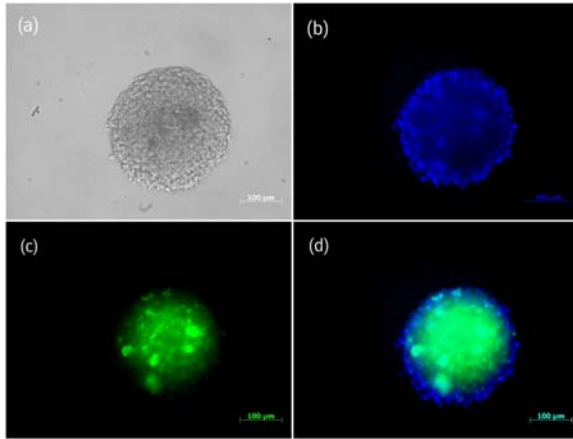
Fig. 3: Nestin immunocytochemistry of neurosphere. Observation concluded that virtually most of the cells within the sphere are positive for nestin
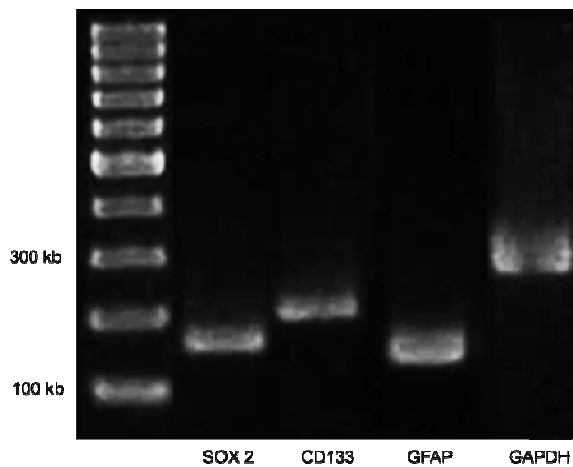

Fig. 4: Expression of Sox2, CD133, and GFAP in neurospheres were evaluated by RT-PCR and electrophoresis on gel

Discussion

Culture of NSCs

Since there was only a quite small percentage of NSCs in adult brain (less than 0.1%) (Reynolds et al., 1992), foetal brain became a promising source for NSC harvesting. In early phases of neural tissue genesis, radial glial cells represented the major cell type in the neural tube and comprise the neurogenic population of the central nervous system. NSC in the adult SVZ was derived from embryonic radial glial cells. Neural stem cells isolated from E14 fetus had been shown to proliferate in response to EGF and FGF2 to give rise to neurospheres. E14 NSCs also had the classical properties of self-renewal and multi-potentiality (Reynolds et al., 1992).

When the spheres developed, secondary spheres could be generated from the primary ones. The bigger the

neurospheres, the more easily they attached to the culture surface. When a neurosphere attached to the flask surface, it would trigger differentiation into mature neural cells. Although agarose was used to reduce the probability of cell attachment but it could not prevent attachment of large and heavy neurospheres. On other hand, only the outside cell could absorb nutrients from the medium while the inside cell could not because neurospheres were very compact. Therefore, passaging was very important to control cell differentiation. Passaging of neural stem cells was not only for providing them more space to proliferate, but also to keep the inside cells in contact with the nutrients. Moreover, passaging could demonstrate self-renewal ability of the neural stem cells via generation of new spheres from single cells.

The neurospheres could be cultivated up to 40-60 days. Some neural sphere could reach the diameter of 1mm before they attach to the surface. After 40-60 days cultivation, the neurosphere slowly disintegrated until all the cells inside are dead. The most serious problem of neural stem cell cultivation was the low stem-cell potential. After long-term culture, neural stem cells could not retain their potential after many replications. Despite of effective and terrific culture medium, supplements were developed to maintain the stem-cell characteristic of neural stem cells; most could not maintain the neurospheres after 90 days culture.

Fibroblast contamination was also a serious problem. Fibroblast was among the well-adapted cell culture condition. They could grow and divide into many culture media. Outspreading of fibroblast was very difficult to control. Since neural stem cells proliferated as floating spheres, fibroblasts could easily be separated through transfer of the cell suspension into a new culture flask.

Enzymatic digestion played a crucial role in primary culture of NSCs. Since NSCs were cultivated as floating multicellular spheres, the enzymatic activity would affect the sphere aggregation ability of the cells. Trypsin was a popular protease for cell isolation, but it would damage membrane of the proteins essential for the sphere formation. Papain was reported to provide a greater yield of viable cells and do less damage to the cells (Hutton et al., 2008).

Nscs stemness analysis

This result shows that our neurospheres expressed nestin. Nestin was reported to be widely expressed in all neural precursor cells (Doyle et al., 2001). Neural differentiation involved down-regulation of nestin and induction of neurofilaments (class IV) in neurons or GFAP (class III) in astrocytes (Chan-Ling et al., 2009; Doetsch et al., 2003; Weiss et al., 1996).

In depth studies on the distribution and expression of nestin in mitotically active cells indicated a complex role in the regulation of the assembly and disassembly of intermediate filaments, which together with other structural
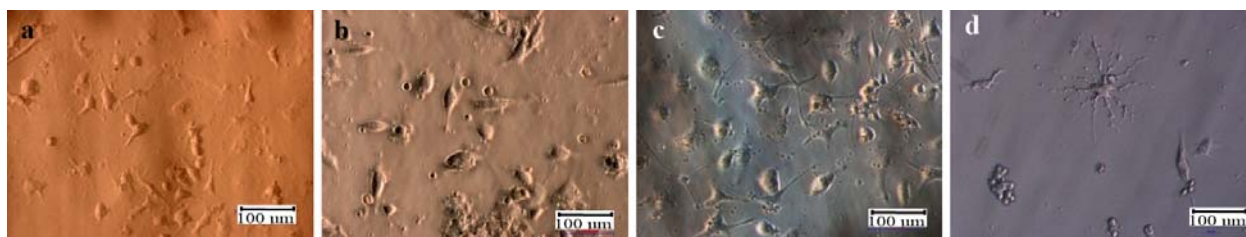
Fig. 5: Astrocyte differentiation of NSC. (a) NSC monolayer culture on poly-L lysine coated flasks. They were oval-shaped and had two dendrites. (b) After 3 days of differentiation, the cell morphology changed a bit. They were shorter, rounder, and some with more dendrites. (c) After 10 days, multi-dendrite cells were formed and after 3weeks, astrocyte-like cell was observed
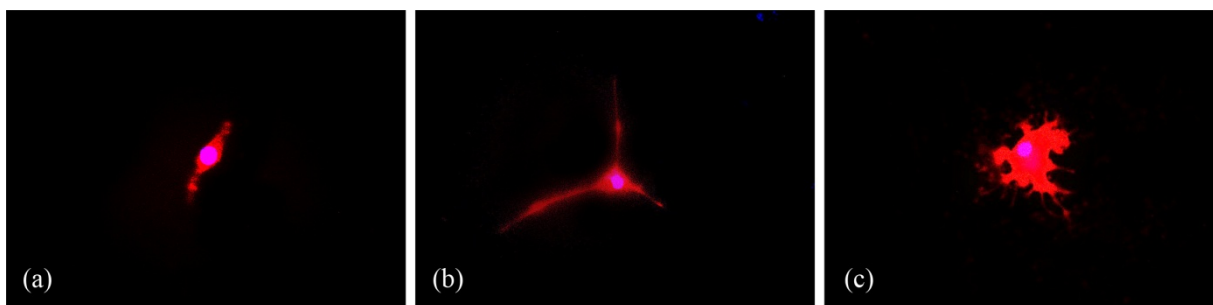

Fig. 6: GFAP immunocytochemistry. Results showed that differentiated cells were positive for GFAP marker

proteins participate in remodeling of the cell. The role of nestin in dynamic cells particularly, structural organization of the cell, appeared strictly regulated by phosphorylation, especially, its integration into heterogeneous intermediate filaments with vimentin or α-internexin (Michalczyk et al., 2005).

The results showed that cells from neurospheres express Sox2, CD133 and GFAP. Sox2 (SRY-sex determining region Y-box 2) was a transcription factor for maintaining self-renewal of stem cells (Tao et al., 2011). CD133, transmembrane glycoprotein, was also a marker for stem cell. The expression of these genes was used to prove that the target cells are stem cells. The expression of Sox2 and CD133 in these candidate cells strengthened the conclusion that these cells were neural stem cells.

Immature astrocyte had oval body (17 – 25µm in diameter), positive for vimentin/GFAP/Pax2 with many orientated dendrites. Mature astrocyte's diameter was 11 – 14µm, negative for vimentin and positive for GFAP and Pax2. Astrocyte differentiation was induced by LIF and BMP2 (Chan-Ling et al., 2009). GFAP is a popular marker to identify astrocytes. GFAP plays important roles in some processes of the CNS (central nervous system) such as cell communication, and the blood-brain barrier. GFAP also regulates the filament network during cell mitosis. GFAP was an intermediate filament protein involved in the structure of astrocyte cells and specifics for these cells. The cells from the neurospheres expressed GFAP because the outermost cells of spheres differentiated into astrocyte precursor cells.

Conclusion

In this study, we showed a successful culture of neural stem cells from the murine foetal brain. The cells were harvested from E14; foetal brain could form spheres after 7 days of culture in free serum medium, supplemented growth factors and some elements that were needed for the development of neural stem cells. The candidate cells isolated from primary culture of NSCs had self-renewal ability. In addition, the candidate cells could differentiate into astrocytes – one type of glial cells in central nervous system (CNS) and expressed neural stem cell marker, nestin. This population of cells was a promising source to study neural stem cells and for the treatment of neurodegenerative disease.

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