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THE DECREASED SELF-RENEWAL POTENTIAL OF NPCs DURING HUMAN EMBRYONIC BRAIN DEVELOPMENT WITH REDUCED ACTIVITY OF MAPKs

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Abstract

Study of neural progenitor cells (NPCs) is important for treatment of degerative diseases in central nervous system. One of the key questions in NPCs transplantation therapy is about the understanding of which stage of the NPCs in brain development is ideal. Herein we investigated survival, proliferation and apoptosis of NPCs from 12 w, 16 w and 20 w human embryonic brain, meanwhile, the phosphorylation of mitogen-activated protein kinases (MAPKs) signaling were analyzed. The results showed that the survival, proliferation and cell division of 16 w and 20 w human NPCs significantly decreased comparing with 12 w human NPCs in vitro; and the NPCs apoptosis remarkably increased. Phosphorylation of ERK1/2 of 16 w and 20 w NPCs significantly decreased comparing with 12 w human NPCs, however phosphorylation of p38 MAPK increased. NPCs proliferation increase when ERK1/2 signaling is activated by PMA.. The results demonstrated that self-renewal potential of NPCs decreased in culture during human embryonic brain development, the activity of ERK signaling pathway were decreased, and suggest NPCs from 12-week fetuses might be better donor for cell transplantation during the period of 12-20 weeks because of their advantage on survival and proliferation.

Key words: Neural progenitor cells; proliferation; apoptosis; Mitogen-activated protein kinases.

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INTRODUCTION

Neural progenitor/stem cells (NPCs) are multipotential progenitor cells that are undifferentiated and capable of self-renewal, proliferation in the mammalian central nervous system (CNS) during development and throughout adulthood. These cells have the potential ability to differentiate into different neural lineages like neurons, astrocytes and oligodendrocytes (1,10,16). NPCs can be isolated and propagated as neurospheres in culture in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (8,28). In recent years, NPCs-based therapy has been successfully repaired for CNS diseases such as nerve injury, ischemic brain, stroke injury and neurodegenerative disorders (5,20,21). But the survival rate and proliferation of human NPCs were low after cell transplantation. A key question in cell transplantation and regenerative therapies is which stage NPCs from human embryonic brain development are ideal in cell transplantation therapies. Therefore, it is important to investigate NPCs self-renewal potential in different stages of human embryonic brain development and the cellular biology processes.

Development of the mammalian nervous system is governed by a complex interplay between cell-extrinsic and cell-intrinsic cues. The extrinsic cues include a complex array of signaling molecules such as extra-cellular matrix (ECM) and growth factors, which can affect and vary the fate decision of NPCs (2,11,27,35). The different members of mitogen-activated protein kinases (MAPKs) family participate in signaling cascades through evolution, which regulate biological activities (26,37). Three major MAPKs members including extracellular signal-related protein kinases (ERK), c-Jun N-terminal protein kinase (JNK) and p38 MAP kinase are involved in developmental neurobiology (3,9,29). For example, it is reported that ERK, JNK and p38 participate in regulating the survival, proliferation, apoptosis and differentiation of NPCs (17,19,24,25,34).

In this experiment, we will compare self-renewal potential of human NPCs in different stages of human embryonic brain development and explore the mechanism related to cell signaling pathways.

MATERIALS AND METHODS

Human NPCs culture and trypan blue staining

Human NPCs were prepared from 12, 16 or 20-week fetuses cortex of selectively terminating normal pregnant women (the 2nd Affiliated Hospital, College of Medicine, Xi'an Jiaotong University). We obtained informed consent from the pregnant woman. The experimental protocols were approved by the Ethics Committee of Xi'an Jiaotong University College of Medicine and followed the guidelines of the Declaration of Helsinki. The methods of specimen collection were conducted in accordance with the guidelines of National Institutes of Health. The cortex was isolated in chilled sterile phosphate-buffered saline (PBS) containing 0.6% glucose under sterile condition. After removal of the meninges, the cortex was mechanically dissociated into single-cell suspensions in serum-free Dulbecco's Modified Eagles's Medium and Hams F12 (DMEM/F12) (Invitrogen, Carlsbad, CA, USA). After centrifugation for 5 min at 800 rpm, the cells were stai-

ned by 0.4% trypan blue (Sigma, St Louis, MO, USA) for 3 min, and were observed using DP71 camera (ver 5.1, Olympus, Japan). The stained cells were counted. Last, the cells were cultured in culture flasks at a density of 100, 000 cells/ml of serum-free DMEM/F12 supplemented with 2% B27, 1% N2, 1% penicillin, 1% streptomycin, 20 ng/ml hEGF, 10 ng/ml bFGF (all from Invitrogen, Carlsbad, CA, USA) and 2.5 μg/ml heparin (Sigma, St Louis, MO, USA). After 6-7 ds of culture in vitro, the primary neurospheres were passaged by mechanical dissociation. The single cell was cultured at a density of 50, 000 cells/ml for 5 ds until neurospheres were formed (passage 1 neurospheres). Passage 1 neurospheres were detected for below experiments in vitro.

MTT assay and diameter measuring of neurospheres

The proliferation of human NPCs from 12, 16 and 20-week fetuses was estimated by utilizing the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based assay. Passage 1 human NPCs from 12, 16 and 20-week fetuses were seeded into 96-well plates (20, 000 cells/well in 200 μl medium), and 20-week NPCs were treated with PMA (phorbol 12-myristate 13-acetate, 100 nM) (Sigma, St Louis, MO, USA) activating ERK1/2, then incubated for 1, 2, 3, 4, 5, 6 and 7 ds at 37 °C in 5% CO_2. At the end of culture, the diameters of neurospheres were measured using DP71 camera (ver 5.1, Olympus, Japan), following 20 μl of 5 mg/ml MTT (Sigma, St Louis, MO, USA) solution was added per well and the cells were incubated for another 4 hours at 37 °C. Supernatants were removed and formazan crystals were dissolved in 150 μL of dimethylsulfoxide (Sigma, St Louis, MO, USA). Finally, optical density was determined at 492 nm using multimicroplate test system(POLARstar OPTIMA, BMG Labtechnologies, Germany). The results were collected as the mean of more than three independent experiments.

Flow cytometry analysis for cell cycle

To detect cell cycle, DNA content per duplicate was analyzed using a flow cytometer. Passage 1 NPCs from 12, 16 and 20-week fetuses were cultured for 3 ds. Then, the cells were dissociated into single-cell suspensions and fixed in 75% ice cold ethanol overnight at 4 °C. The fixed cells were stained with 50 μg/ml propidium iodide (PI) containing 50 μg/ml RNase A (DNase free) for 15 min at room temperature in the dark, and analyzed by fluorescence activated cell sorting (FACSCalibur, BD Biosciences, San Jose, CA, USA). The cells were excited at 488 nm, and the emission was collected simultaneously through a 630 nm filter. 20,000 cells were collected from each sample. We evaluated the changes of cell cycle distribution by calculating the proliferation index (PI) and S-phase cell fraction (SPF). The following formulas were used: PI=(S+G2/M)/(G0/G1+S+G2/M), SPF=S/(G0/G1+S+G2/M) (6).

Hoechst staining

To analyze apoptosis of NPCs, hoechst 33342 labeling was used to assess the chromosomal condensation and morphological changes. Passage 1 NPCs from 12, 16 and 20-week fetuses were seeded into 24-well plates and cultured for 3 ds. Following, the cells were dissociated into single-cell suspension. Then, the single cell was plated onto the poly-L-lysine-coated coverslips at a concentration of 50, 000 per ml per well in 24-well plates for another 6 hours. The cells were fixed in 4% paraformaldehyde for 1 hour. Cells were stained with Hoechst 33342 (5 μg/ml) (Sigma, USA). The stained cells were observed under UV illumination using an Olympus fluorescent microscope. The percentage of apoptotic cells were determined by counting the number of nuclear condensation cells versus the total cells in each experimental condition. The results were collected as the mean of three independent experiments.

Western blot analysis

To analyze the expression of phosphorylated extracellular signal regulated kinase (ERK), c-Jun N-terminal protein kinase (JNK) and p38, passage 1 human NPCs from 12, 16 and 20-week fetuses were cultured for 3 ds. Then, the cells were lysed in RIPA lysis buffer. Insoluble material was removed by centrifugation at 12, 000 rpm for 10 min at 4 °C. Cell lysates were subjected to electrophoresis using 12% SDS polyacrylamide gels and transferred to a nitrocellulose membrane. Membranes were blocked for 2 hours in 5% non-fat dry milk in PBS. The membrane was incubated with primary monoclonal antibodies overnight at 4 °C and secondary antibody for 4 hours at room temperature. These antibodies were as follow: rabbit monoclonal anti-ERK1/2 (1:2000, Cell Signaling, Danvers, MA, USA), mouse monoclonal anti-P-ERK 1/2 (1:2000, Cell Signaling, Danvers, MA, USA), rabbit polyclonal anti-JNK2 (1:1000, Santa Cruz, CA, USA), mouse monoclonal anti-P-JNK (1:2000, Cell Signaling, Danvers, MA, USA), Rabbit polyclonal anti-p38 (1:2000, Cell Signaling,Danvers, MA, USA), rabbit polyclonal anti-P-p38 (1:1000, Cell Signaling, Danvers, MA, USA), mouse monoclonal anti-β-Actin (1:5000, Santa Cruz, CA, USA). For chemiluminescence detection, these membranes were incubated in the dark with ECL (Amersham). The luminescent signal was recorded and quantified with the Syngene G Box (Syngene, UK). Results were collected as the mean of three independent experiments.

Statistics analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA). Tukey's post-hoc analyses were used to determine the difference between groups. All the quantitative data were presented as mean ± SEM. The $P < 0.05$ was considered statistically significant.

RESULTS

The change of survival and proliferation of human NPCs from different stages of embryonic brain

To determine the change of survival of human NPCs from 12, 16 and 20-week fetuses, the primary cultured human NPCs were stained by 0.4% trypan blue. The results showed that the cell survival rate from 12, 16 and 20-week fetuses NPCs were 82.85±4.11%, 53.93±5.03% and 18.27±5.16%, respectively (Fig. 1A). The cell survival of NPCs significantly decreased with human embryonic brain development ($P < 0.05$). MTT assay was used to analyze the cell activity and diameter measuring of neurospheres method was employed to represent the cell growth. MTT assay showed that the activity of 16 w and 20 w human NPCs significantly decreased comparing with 12 w human NPCs in vitro, and the activity of 20 w human NPCs remarkably diminished comparing with 16 w human NPCs at 3, 4, 5, 6 and 7 ds (Fig. 1B) ($P < 0.05$). The diameter

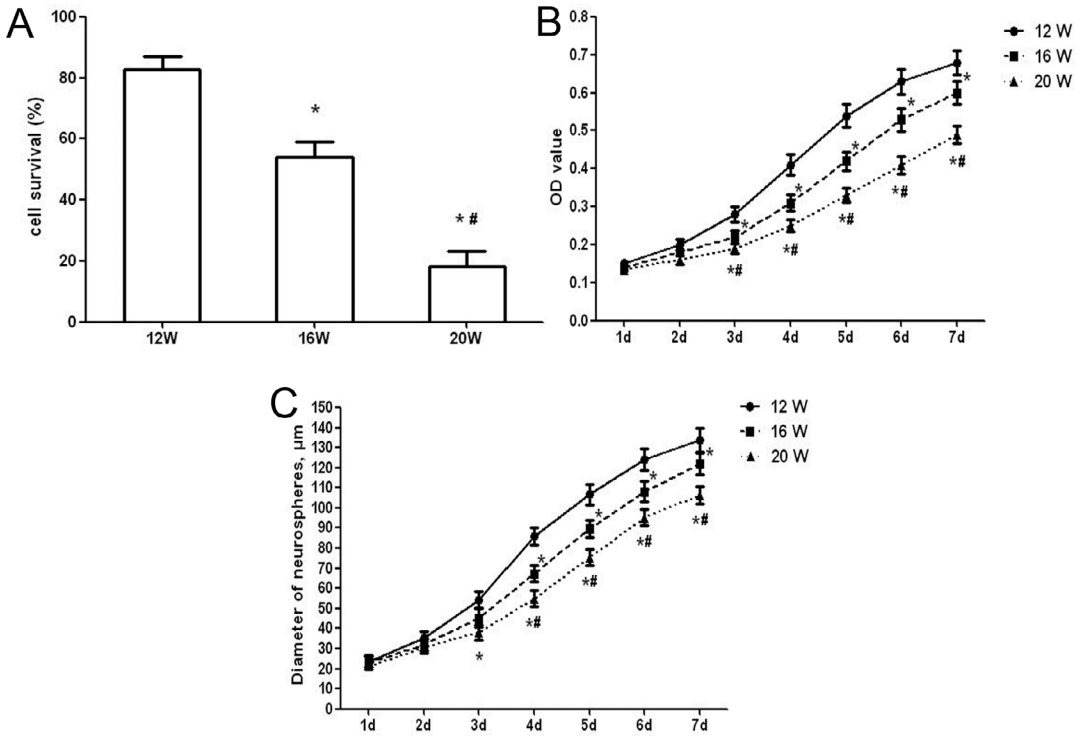

Figure 1. The survival and proliferation of brain NPCs from different stages of human embryo. (A) Trypan blue staining showed that the survival rate of primary human NPCs gradually decreased with embryonic brain development. (B) MTT assay showed that the activity of human NPCs from 12 w, 16 w and 20 w embryonic brains gradually descend with embryonic brain development. (C) The neurospheres diameters of NPCs from 12 w, 16 w and 20 w embryonic brain gradually decreased with embryonic brain development (* $P < 0.05$, compared with 12 w NPCs; # $P < 0.05$, compared with 16 w NPCs, n=5).

measuring of neurospheres showed that the mean diameter of neurospheres of 16 w and 20 w human NPCs significantly diminished comparing with 12 w human NPCs in vitro, and the diameter of neurospheres of 20 w human NPCs remarkably decreased comparing with 16 w human NPCs at 4, 5, 6 and 7 ds (Fig. 1C) ($P < 0.05$).

The change of cell cycle of human NPCs from different stages of embryonic brain

As the cell cycle is involved in the regulation of cell growth, these processes were examined using a flow cytometer 3 d after cultured. As illustrated in Fig. 2, PI and SPF were significantly decreased in 16 w and 20 w human NPCs groups comparing with 12 w human NPCs groups in vitro, and remarkably diminished in 20 w human NPCs groups comparing with 16 w human NPCs groups (Fig. 2) ($P < 0.05$).

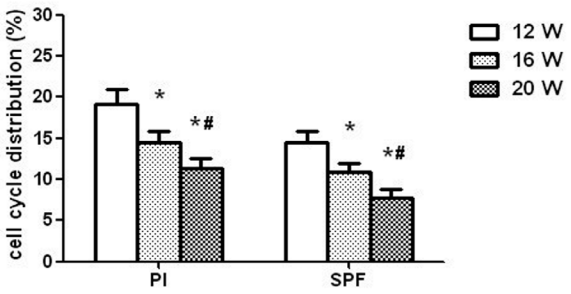

Figure 2. The cell cycle of different stages human embryo NPCs. Cell cycle analysis showed that PI and SPF gradually decreased with embryonic brain development. Proliferation index (PI)=(S+G2/M)/(G0/G1+S+G2/M), SPF=S/(G0/G1+S+G2/M) (* $P < 0.05$, compared with 12 w NPCs; # $P < 0.05$, compared with 16 w NPCs, n=5).

The diversity of apoptosis of human NPCs from different stages of embryonic brain

To detect the difference of cell death of 12 w, 16w and 20 w human NPCs, we observed the apoptotic morphology of nuclei in human NPCs with Hoechst staining. The proportion of apoptotic nuclei of 12 w, 16w and 20 w human NPCs was 8.91±1.44%, 14.55±1.64% and 23.49±2.33%, respectively (Fig. 3). The proportion of apoptotic nuclei of 16w and 20 w human NPCs remarkably increased comparing with 12 w human NPCs in vitro, and significantly increased in 20 w human NPCs groups comparing with 16 w human NPCs groups ($P < 0.05$).

The change of phosphorylation of MAPK signaling pathway in human NPCs

To explore the possible molecular mechanisms of human NPCs proliferation decreases with human embryonic brain development, 12 w, 16w and 20 w human NPCs were cultured or 3 ds. Then, the expression of phosphorylated ERK1/2, JNK2 and p38 was measured. There were no significant difference in the total expression of each signaling pathway (ERK1/2, JNK2 and p38), but the phosphorylation expression of three MAPK signaling molecules evidently changed in all kinds of groups. The ratio of p-MAPK/total MAPK was used to indicate the phosphorylation and activation of the proteins. The p-ERK1/2 level of 16w and 20 w human NPCs remarkably decreased comparing with 12 w human NPCs, and significantly diminished in 20 w human NPCs comparing with 16 w human NPCs groups (Fig. 4A) ($P < 0.05$). However, there were no significant changes in p-JNK2 level (Fig. 4B). But p-p38 level of 16w and 20 w human NPCs significantly increased comparing with 12 w human NPCs, and remarkably increased

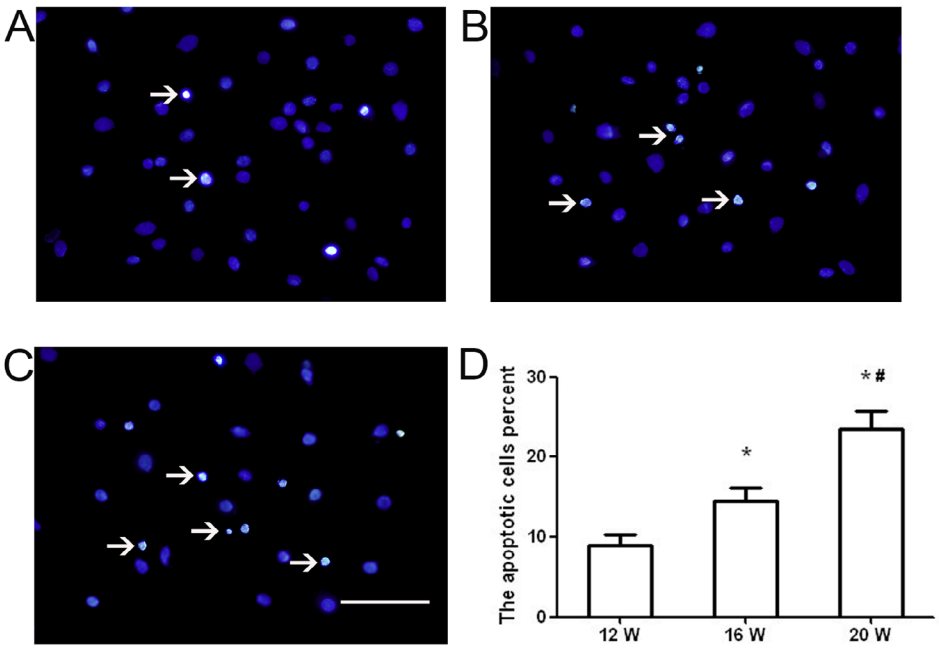

Figure 3. The cell death of brain NPCs from different stages of human embryo by nuclei stained with Hoechst 33342. Panel (A–C) showed Hoechst 33342-stained apoptotic cells in 12 w, 16 w and 20 w human NPCs, respectively. Panel D showed the statistical percentage of apoptotic cells in all groups (* $P < 0.05$, compared with 12 w NPCs; # $P < 0.05$, compared with 16 w NPCs, n=3). The apoptosis gradually increased with embryonic brain development. White arrowheads indicate apoptotic cells. Scale bar=100 μm.

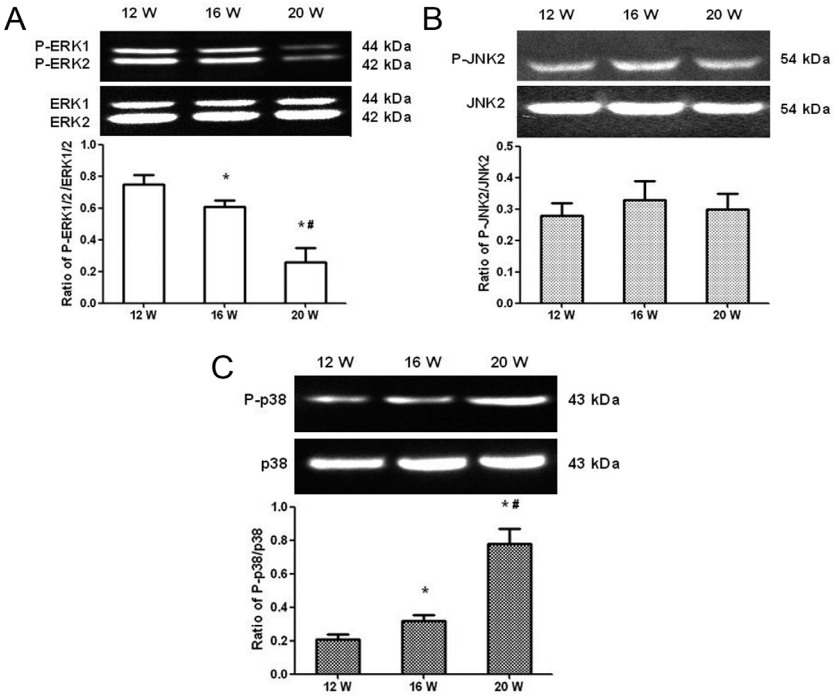

Figure 4. Expression of phosphorylated ERK1/2, JNK2 and p38 in brain NPCs from different stages of human embryo. (A) Phosphorylated ERK1/2 significantly decreased with embryonic brain development. (B) Phosphorylated JNK2 had no changes with embryonic brain development. (C) The expression of phosphorylated p38 remarkably increased with embryonic brain development (* $P < 0.05$, compared with 12 w NPCs; # $P < 0.05$, compared with 16 w NPCs, n=3). Data were expressed as a ratio of the normalized percentage of p-MAPKs and MAPKs.

in 20 w human NPCs comparing with 16 w human NPCs groups (Fig. 4C) ($P < 0.05$).

NPCs self-renewal potential increase when ERK1/2 signaling is activated

MTT assay showed that the activity of 20 w+PMA (100 nM) human NPCs significantly increased comparing with 20 w human NPCs in vitro at 3, 4, 5, 6 and 7 ds ($P < 0.05$), and there were no significant changes in the activity comparing with 12 w human NPCs at 3, 4, 6 and 7 ds (Fig. 5A) ($P > 0.05$). The diameter measuring of neurospheres showed that the mean diameter of neurospheres of 20 w+PMA human NPCs remarkably increased comparing with 20 w human NPCs at 3, 4, 5, 6 and 7 ds ($P < 0.05$), and there were no significant changes in diameter of neurospheres comparing with 12 w human (Fig. 5B) ($P > 0.05$).

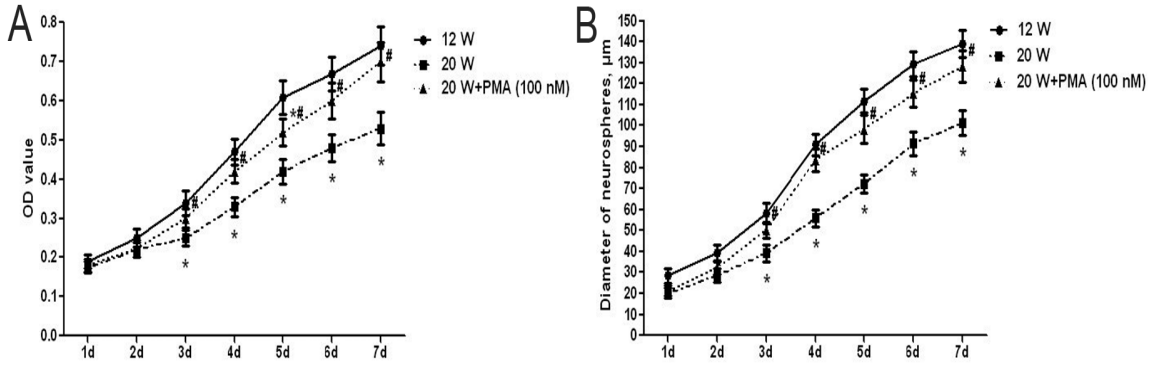

Figure 5. Effect of ERK1/2 activation on proliferation of human NPCs. (A) MTT assay showed that the activity of human NPCs from 20-week embryonic brains increased when ERK1/2 is activated by PMA (100 nM). (B) The neurospheres diameters of NPCs from 20 w embryonic brain increased after ERK1/2 is activated by PMA (100 nM). (* $P < 0.05$, compared with 12 w NPCs; # $P < 0.05$, compared with 20 w NPCs, n=3).

DISCUSSION

In the course of vertebrate nervous system development, NPCs have the capacity for self-renewal and generation of new neurons, astrocytes and oligodendrocytes supporting cells. They are present not only in the fetal brain but also in the newborn and adult special brain areas (23). The proliferation and neuronal differentiation of NPCs begins with the activation of membrane receptors by extracellular signals. These receptors activate intracellular signaling cascades that lead to changes in transcription of genes that are essential for proliferation and differentiation of NPCs (4). Some genes are common to both young and old NPCs, others are expressed only at certain time points. Temporally specific genes may coordinate an developmental program, so NPCs produce certain progeny at certain times and the self-renewal potential of NPCs gradually decreases (30). In this study, we found that the cell survival and proliferation of NPCs significantly decreased in vitro with human embryonic brain development. And human NPCs from 12-week fetuses cortex were ideal donor for cell transplantation therapy because of their advantage on survival and proliferation at this period.

The gap (G1) phase of cell cycle is an unique period when cells respond to environmental signals to determine cell fate such as survival, proliferation, differentiation and cellular senescence (31). There are many important cell cycle regulators, including cyclinD, cyclinE, Cdk4 and Cdk6 protein kinase complexes, which can govern the cellular progression through the G1 phase of the cell cycle (32). In this experiment, we demonstrated that there were more cells crossing G1/S node and entering into cell cycle in human NPCs from 12-week fetuses, resulting in the increasing of 12 w human NPCs proliferation.

ERK, JNK and p38 MAP kinase are three main members of MAPKs family, they play an important role in the CNS development and differentiation (36). The ERK cascade could be activated by growth factors and transmit signals to promote cell proliferation and survival (14,22). For example, activation of adenosine A1 receptor stimulated proliferation of NPCs occurs via MEK/ERK signaling pathways (13). bFGF plays a key role in regulating proliferation and maintenance of NPCs, which are in part mediated by MEK/ERK signaling pathways (18). It was reported that ERK is required both for the proliferation of neural stem cells in the VZ during embryonic development and in the maintenance of NPC multipotency by suppressing the commitment of these cells to a glial lineage (25). Wang Bin demonstrated that ERK1/2 played an important role in the interplay between cell-extrinsic cues and cell-intrinsic genetic mechanisms in neural stem cell biology (34). Our team previous study also showed: Activation of mGluR7 promotes the proliferation and differentiation of neural progenitor cells with changes in phosphorylation of ERK1/2 signaling pathways (33). So, we further tested the activation of ERK1/2 signaling molecules and found phosphorylation and activation of ERK1/2 gradually decreased with human embryonic brain development, and NPCs self-renewal potential increase when ERK1/2 signaling is activated by PMA. It was also found that hypoxia promoted proliferation of rat NPCs by activating JNK in vitro (6). But we found that there were no significant changes in phosphorylation of JNK2 with human embryonic brain development.

In addition, the phosphorylation of p38 MAP kinase could induce cell apoptosis, and inhibition of p38 with selective inhibitor SB203580 can reduce the cell death (7,12). It was reported that inhibitors of p38 MAPK promoted proliferation of rat NPCs, and and p38 MAPK may be an intrinsic negative regulator of NSC proliferation during early brain development (17). The study of Jeesun also suggested that p38 MAPK signaling act as a negative regulator of NPC proliferation in response to oxidative stress (15). In this study, our results showed that apoptosis of NPCs gradually increased by augmenting of phosphorylation of p38 MAPK with human embryonic brain development.

In summary, we have found that the survival and proliferation of NPCs from human fetuses gradually decreased with human embryonic brain development in vitro, but apoptosis of NPCs gradually increased. During these processes, phosphorylation and activation of ERK1/2 gradually decreased with human embryonic brain development, however phosphorylation of p38 MAPK gradually increased. The results suggest that human NPCs from 12-week fetuses cortex might be better donor for cell transplantation therapy during the period of 12-20 weeks because of their advantage on survival and proliferation. Ne-

vertheless, cell transplantation are far more complex, more work is needed to explore the difference of NPCs survival, proliferation and differentiation in different stages of human embryonic brain development in vivo.

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