Observational Study

Medicine®

OPEN

# Effect of mGluR7 on proliferation of human embryonic neural stem cells

Jing Zhang, PhD, Junmei Zhao, MD, Yani Chen, MD, Haiyan Shi, MD, Xiaoyong Huang, MD, Yanfeng Wang, MD, Yu Wang, MD, Yameng Wei, MD, Wanjuan Xue, MD, Jiming Han, PhD*

**Abstract**

This study is to investigate the effect of metabotropic glutamate receptor 7 (mGluR7) on the proliferation of human embryonic neural stem cells (NSCs) and its molecular mechanism.

Human embryonic NSCs were isolated. The pCMV2-GV146-GFP-mGluR7 plasmid was transfected to over-express mGluR7 while mGluR7 siRNA was transfected to knockdown mGluR7. MTT assay was used to analyze cell proliferation. Flow cytometry was used to detect cell cycle and apoptosis. Protein and mRNA levels were analyzed by Western blot and RT-qPCR, respectively.

The viability of human NSCs and the diameter of neurospheres after 24 hours, 48 hours, and 72 hours of transfection significantly increased by mGluR7 overexpression whereas significantly decreased by mGluR7 knockdown. Ki-67 expression was up-regulated by mGluR7 overexpression whereas down-regulated by mGluR7 siRNA, indicating a promotive effect of mGluR7 on NSC proliferation. After mGluR7 overexpression, G1/G0 phase cell ratio dropped significantly compared with control group, while the S phase cell ratio increased. mGluR7 silencing arrested human NSCs at G1/G0 phase. After 48 hours of transfection, there was a decrease of apoptosis by mGluR7 overexpression, while mGluR7 silencing induced apoptosis of human NSCs. Additionally, overexpression of mGluR7 up-regulated the expression of p-serine/threonine kinase (AKT), cyclin D1, and cyclin-dependent kinase 2 (CDK2). The mGluR7 knockdown had opposite effects. Similarly, mGluR7 down-regulated the expression of Caspase-3/9, while the mGluR7 knockdown promoted this.

mGluR7 can promote the proliferation of human embryonic cortical NSCs in vitro. This effect may be mediated by promoting cell cycle progression, inhibiting cell apoptosis, activating the AKT signaling pathway, and inhibiting the Caspase-3/9 signaling pathway.

**Abbreviations:** AKT = serine/threonine kinase, bFGF = basic fibroblast growth factor, CDK2 = cyclin dependent kinase 2, CNS = central nervous system, EGF = epidermal growth factor, ERK1/2 = extracellular signal regulated protein kinase 1/2, MAPK = mitogen-activated protein kinase, mGluR7 = metabotropic glutamate receptor 7, mGluRs = metabotropic glutamate receptors, NSCs = neural stem cells, PI3K = phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha.

**Keywords:** apoptosis, cell cycle, cell proliferation, metabotropic glutamate receptor 7, neural stem cells

## 1. Introduction

Neural stem cells (NSCs) are pluripotent and self-renewing cells that reside in the central nervous system (CNS) of mammals.[1–3] NSCs can be isolated from embryos and adult brains and develop into neurospheres in vitro in the presence of mitogens such as epidermal growth factor (EGF)[4] and basic fibroblast growth factor (bFGF).[5] They have the potential to differentiate into neurons, astrocytes, and oligodendrocytes.[6] Treatment with NSCs can improve neurological function of cerebral ischemia, nerve damage, and neurodegenerative disorders.[7] However, the molecular mechanism regulating the proliferation of NSCs remains unclear.

Glutamate, a major excitatory neurotransmitter in the CNS, may be associated with neurogenesis.[8] Brain damages such as cerebral ischemia and epilepsy, involve glutamate excitotoxicity but glutamate can also stimulate neurogenesis.[9] Glutamate receptor signaling may regulate neurogenesis after brain injury.[10] Metabotropic glutamate receptors (mGluRs), including mGluR1-8, belong to the family of G protein-coupled receptors and may regulate the proliferation of NSCs in the human brain.[11] The metabotropic glutamate receptor 7 (mGluR7) is the most conserved subtype of the mGluR family. It is expressed in different regions of brain, especially in the cerebral cortex, hippocampus, and cerebellum. It plays a role in the regulating the proliferation of NSCs during development or after nerve injury. For example, our team previously reported[12] that activation of mGluR7 with agonists can promote the survival, proliferation, and differentiation of rat NSCs in vitro by activating the mitogen-activated protein kinase (MAPK) signal transduction pathway. Extracellular signal-regulated protein kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 MAPKs are major members of the MAPKs family. The ERK1/2

Editor: Aleph Prieto.

ZJ and ZJ contributed equally to this article.

This work was supported by the Shaanxi Innovative Talents Promotion Plan-Science and Technology New Star Project (2017KJXX-20), Shaanxi Social Development of Science and Technology Project (2016SF-190), and Yan'an City Science and Technology Research Development Planning Project (2016KS-06) and Scientific Research Plan Projects of Shaanxi Provincial Department of Education (15JK1838).

The authors report no conflicts of interest.

Department of Clinical Medicine, Medical College of Yan'an University, Yan'an, P. R. China.

* Correspondence: Jiming Han, Department of Clinical Medicine, Medical College of Yan'an University, No. 38, Guanghua Road, Yan'an 716000, P. R. China (e-mail: yadxsci@163.com).



Medicine (2019) 98:9(e14683)

Received: 1 May 2018 / Received in final form: 23 January 2019 / Accepted: 28 January 2019

http://dx.doi.org/10.1097/MD.0000000000014683

cascade can transduce both intracellular and extracellular signaling and regulate the cellular activity and gene transcription, thus regulating the structure and function of CNS.[13] However, there may be other mechanisms underlying the regulatory effect of mGluR7 on the proliferation of NSCs, which still need further investigation.

In this study, we aimed to investigate the effects of mGluR7 on the proliferation of human embryonic NSCs in vitro and its molecular mechanisms.

## 2. Materials and methods

### 2.1. Isolation and culture of human embryonic brain cortex NSCs

Human embryos (n=6) were provided by the Department of Obstetrics and Gynecology of the Affiliated Hospital of Yan'an University. Informed consent was signed by patients and their families. The study was approved by the ethics review board of Yan'an University.

The 12~16w aborted embryos were taken and immediately placed in saline at 4°C. Under sterile conditions, cerebral cortex was isolated on ice and washed with DMEM/F12 basic medium twice and PBS once. The isolated brain cortex was made into single cell suspension by pipette and then filtered with a 400-mesh screen (pore-size 38 μm; Shanghai solarbio Bioscience & Technology Co., LTD, Shanghai, China). The harvested cell suspension was centrifuged at 800 rpm for 8 min. After discard of the supernatant, the cells were washed twice with basal medium and resuspended in 3 ml of complete medium, which was DMEM/F12 basal medium (Gibco, Grand Land, NY) supplemented with 10 ng/mL bFGF (Gibco), 20 ng/mL human EGF (Sigma Chemical Co., St. Louis, MO), 1 × N2 supplement (Gibco), 1 × B27 supplement (Gibco), 100 U/mL penicillin, 100 U/mL streptomycin, and, 0.4 U/mL heparin. The isolated NSCs were identified by Nestin expression.

Approximately $8 \times 10^5$ NSCs were seeded into a T50 flask (Labserv) and cultured in at incubator at 37°C with 5% $CO_2$. Complete medium (1 mL) was added to each bottle the next day followed by medium change every other day. After 5 to 7 days of culture, they were sub-cultured.

### 2.2. The transfection of mGluR7 siRNA and mGluR7 expression plasmid

The 1 passage NSCs were used for transfection. For siRNA transfection, the siRNA was synthesized by Shanghai GenePharma Co., Ltd. The sequences were as follows: human mGluR7 siRNA (sense 5′-GAAGACACAGAAAGGAACUTT-3′, antisense 5′-AGUUCCUUUCUGUGUCUUCTT-3′) and negative siRNA (NC-siRNA, sense 5′-UUCUCCGAACGUGUCACGUTT-3′, antisense 5′- CGUGACACGUUCGGAGAATT-3′). The siRNA transfection was performed with Lipofectamine 2000 (Invitrogen). Briefly, Lipofectamine 2000 and siRNA (60 nM) was mixed with DMEM/F12 basal medium, respectively, and incubated for 5 min at room temperature. Then, the 2 solutions were mixed and incubated for 15 minutes at room temperature. The siRNA-Lipofectamine 2000 mixture was added to the culture plate. For plasmid transfection, the pCMV2-GV146-GFP-mGluR7 expression plasmid or pCMV2-GV146-GFP empty vector (constructed by Sangon Biotech, Shanghai, China) was transfected into NSCs with TurboFect™ (Thermo Scientific, Basingstoke, UK), according to the manufacturer's instructions. Cells transfected with NC-siRNA or pCMV2-GV146-GFP empty vector were used as control groups. Cells were cultured for 24 hours, 48 hours, and 72 hours, respectively, after siRNA or plasmid transfection.

### 2.3. Real-time fluorescence quantitative PCR

Total RNA was extracted from cells with Trizol and reverse transcribed into cDNA. The mRNA level of mGluR7 and β-actin was detected with real-time fluorescence quantitative PCR. The primer sequences were: mGluR1-F 5'-CCTCTGTATCGCCCATTCTGA-3' and mGluR1-R 3'-GGAAGCCTCTCTCGGAGTTT-5'; mGluR2-F 5'- CACGGCAGTGTGTACCTTACG-3' and mGluR2-R 3'- GATGCGTGCAATGCGGTTG-5'; mGluR3-F 5'-GGGGCCTGTTTCCTATTAACG-3' and mGluR3-R 3'- TCACTCCTGGTAGCAAGTAATCA-5'; mGluR4-F 5'- CACCTTAGAATAGAGCGGATGC-3' and mGluR4-R 3'- TGTCATAGGGACACGTCTTACA-5'; mGluR5-F 5'- AACTGGACCTATGTATCAGCCG-3' and mGluR5-R 3'- GGCGATGCAAATCCCTTCC-5'; mGluR6-F 5'-GCCTCCGAGGGCAACTATG-3' and mGluR6-R 3'- GGGAATCTTGATAGACTGGGCAA-5';mGluR7-F 5'-CTGTTGGAGAGAGCGAGCAG-3' and mGluR7-R 5'- AGAGAGGGTGAGGGGTCC-3'; mGluR8-F 5'-CCTATGCTTTGGAGCAGTCTC-3' and mGluR8-R 3'- CATTAGCACACTTCACATCCGAA-5'; and β-Actin-F 5'-TGGCACCCAGCACAATGAA-3' and β-Actin-R 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'. The PCR system included 12.5 μL of SYBR Green PCR Master Mix, 1 μL of upstream primer (10 μM), 1 μL of downstream primer (10 μM), 2 μl of cDNA (100 ng) and 8.5 μL of $ddH_2O$. The reaction was carried using SYBR Premix Ex Taq™II (TaKaRa) with 1-minute predenaturation at 95° C and 40 cycles of 10 seconds denaturation at 95° C, annealing at 60° C, and 40 seconds extension. β-actin was selected as internal reference. The mGluR7 mRNA expression was calculated with $2^{-\Delta\Delta Ct}$ method.

### 2.4. MTT assay and neurosphere diameter measurement

The 1 passage NSCs were seeded into 96-well plates with 20,000 cells per well. The cells were grouped and transfected with mGluR7 siRNA and mGluR7 expression plasmid as above described.

For MTT assay, after culturing for 24 hours, 48 hours, and 72 hours, respectively, MTT was added and incubated at 37°C for 4 hours until purple precipitate was visible. Then DMSO was added after removing the culture supernatant. The plate oscillated for 10 minutes until purple precipitate was dissolved. The absorbance was measured at 492 nm with a microplate reader.

For neurosphere diameter measurement, after culturing for 24 hours, 48 hours, and 72 hours, respectively, cells were photographed with Image-Pro Express software 7.0 (ImageNet Express Pro) and the diameters of neurospheres were measured.

### 2.5. Flow cytometry analysis

The 1 passage NSCs were seeded into 6-well plates with 20,000 cells per well. The cells were grouped and transfected with mGluR7 siRNA and mGluR7 expression plasmid as above described. For cell cycle analysis, after 24 hours of transfection, cells were collected, washed with PBS and fixed in 70% ethanol. Then, cells were stained with 0.5 mL of PI (100 μg/mL) at room temperature for 20 minutes in the dark and analyzed with flow cytometry (FACSCalibur, BD Biosciences, San Jose, CA). For

apoptosis analysis, cells were collected at 48 hours after transfection. Then, 5 μL of Annexin V-FITC was added and incubated at 4° C for 15 minutes. Finally, 10 μL of PI was added and incubated at 4°C for 5 minutes before apoptosis analysis.

### 2.6. Western blot

Cells were collected at 48 hours after transfection. The total protein was extracted from cells with RIPA lysis buffer. Proteins were separated by electrophoresis and transferred to PVDF membrane. After blocking with 5% skimmed milk, the membrane was incubated with primary antibodies of rabbit anti-mGluR7, rabbit polyclonal anti-Nestin, rabbit polyclonal anti- Ki67, mouse anti-cyclin D1, rabbit monoclonal anti-cyclin dependent kinase 2 (CDK2), rabbit polyclonal anti- serine/threonine kinase (AKT), mouse monoclonal anti-p-AKT, rabbit polyclonal anti-Caspase-3, rabbit polyclonal anti-Caspase-9, and anti-β-actin at 4°C overnight. β-actin was used as internal reference. All primary antibodies were purchased from Santa Cruz Biotechnology, USA. After that, the secondary antibody (goat anti-rabbit or goat anti-mouse IgG, Santa Cruz Biotechnology) was added and incubated at room temperature for 1.5 hours. After washing, the membrane was developed.

### 2.7. Statistical methods

SPSS 18.0 was used for data analysis. All data were expressed as mean ± standard error of mean (SEM). Each experiment was repeated 3 times. The *t* test was performed to analyze the differences. $P < .05$ was considered statistically significant.

## 3. Results

### 3.1. The expression changes of mGluR7 after overexpression or silencing

To detect the expression of Nestin (a cell-specific marker of NSC) Western blot was performed. As shown in Figure 1A, Nestin was expressed in all the isolated cells from 6 human embryos, indicating that the isolated cells were identified as NSCs. Western blot was also used to analyze the mRNA levels of other mGluR subtype (mGluR1-6 and mGluR8) after the mGluR7 plasmid transfection. The results showed that there were no significant differences in the mRNA expression levels of other mGluR subtypes after the plasmid transfection (Fig. 1 B). To detect the level of mGluR7 after mGluR7 siRNA and mGluR7 expression plasmid transfection, qRT-PCR and Western Blot methods were performed. The results showed that the expression of mGluR7 mRNA in the cells transfected with the mGluR7 expression plasmid was significantly upregulated compared to the control (Fig. 1 C, $P < .01$). And the mGluR7 mRNA levels in the cells transfected with mGluR7 siRNA were significantly down-regulated than NC-siRNA control. Similarly, mGluR7 protein level was increased after mGluR7 expression plasmid transfection whereas decreased after mGluR7 siRNA transfection. Furthermore, there was still Nestin expression in NSCs after

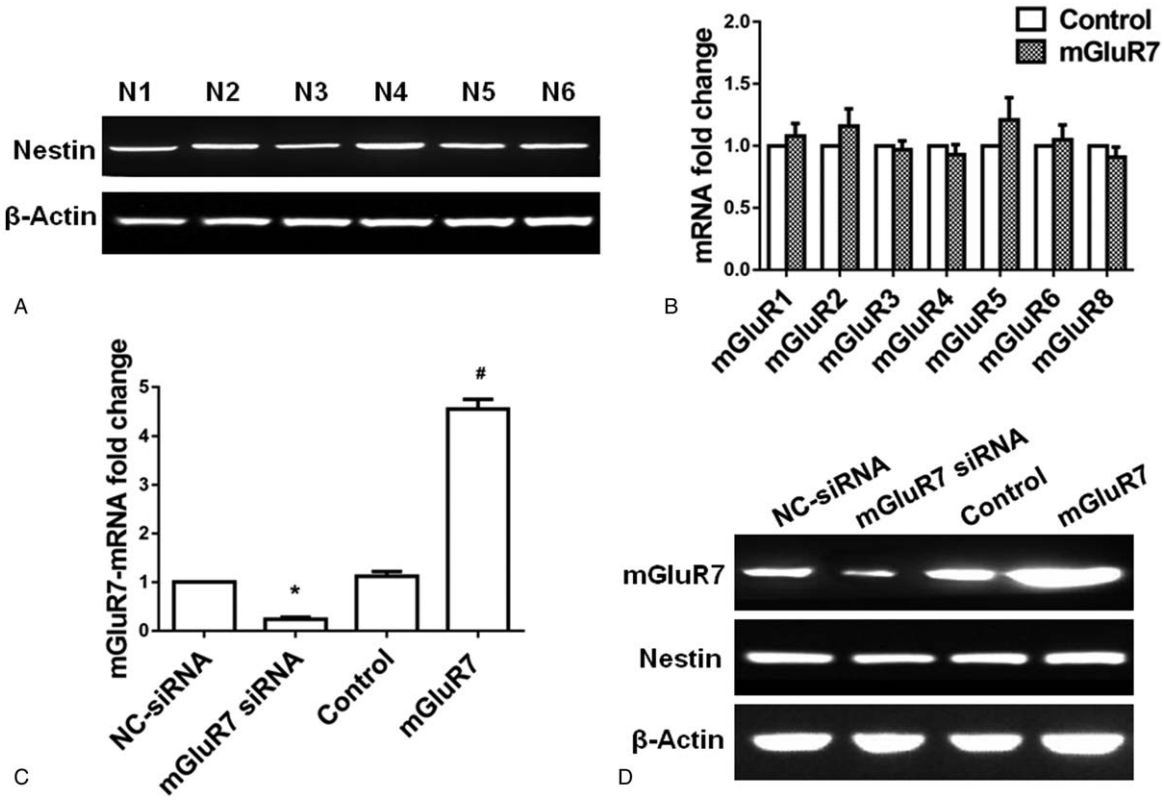


**Figure 1.** Identification of NSCs and analysis of mGluR expression in human NSCs. (A) Western blot was used to detect Nestin expression in the isolated NSCs (N1-N6). NSCs were transfected with pCMV2-GV146-GFP-mGluR7 plasmid and pCMV2-GV146-GFP empty vector (control) or mGluR7 siRNA and NC-siRNA (control). Levels of mRNA and protein were analyzed with qRT-PCR and Western blot. (B) mRNA levels of other mGluR subtypes (mGluR1-6 and mGluR8) after the pCMV2-GV146-GFP-mGluR7 plasmid transfection in the NSCs. (C) Level of mGluR7 mRNA. (D) Protein level of mGluR7 and Nestin. *, compared with NC-siRNA (negative control) group, $P < .01$; #, compared with control (empty vector) group, n = 3, $P < .01$. mGluRs = metabotropic glutamate receptors, mGluR7 = metabotropic glutamate receptor 7, NSCs = neural stem cells.

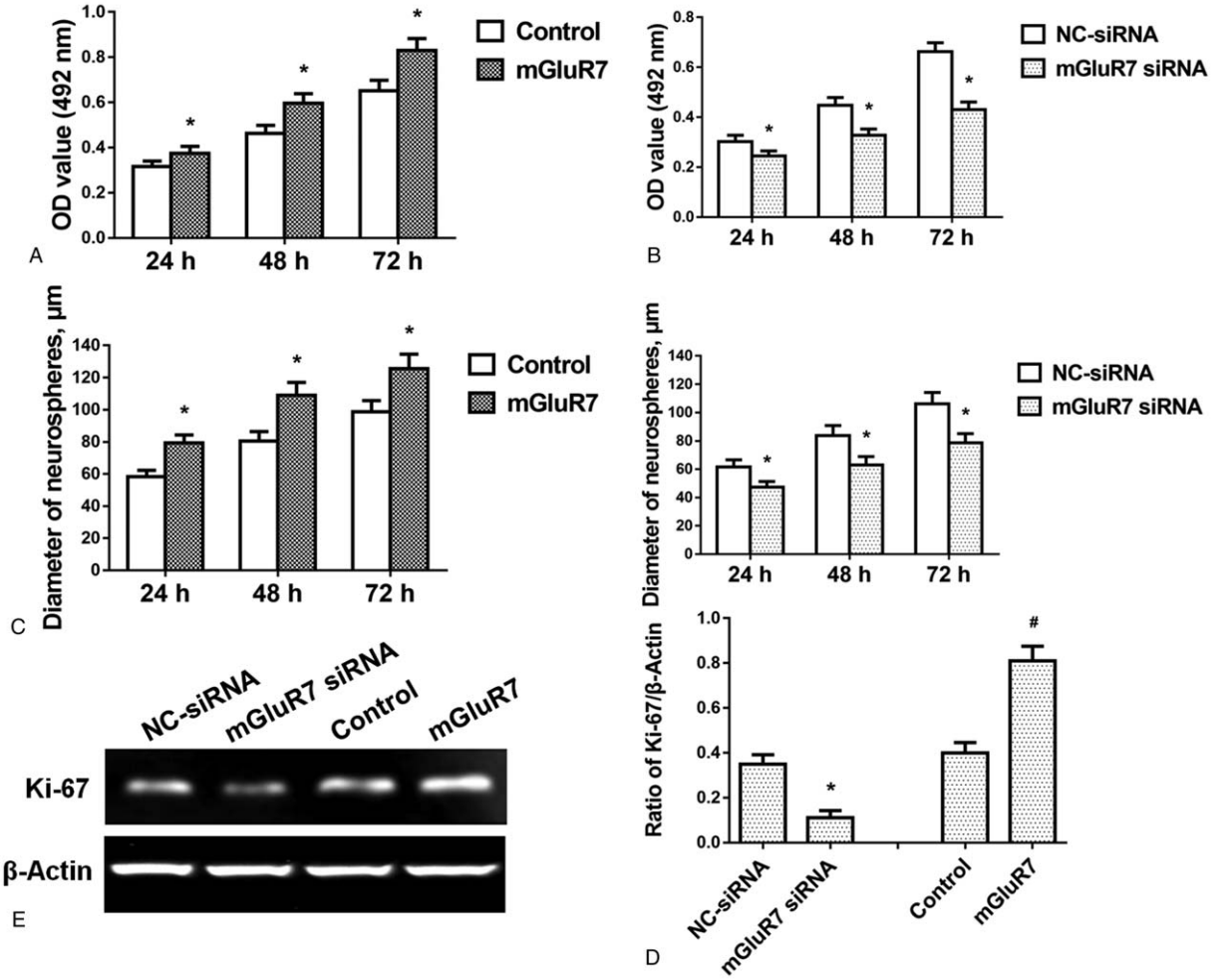


**Figure 2.** Effects of mGluR7 on the proliferation of human NSCs and neurospheres. NSCs were transfected with pCMV2-GV146-GFP-mGluR7 plasmid and pCMV2-GV146-GFP empty vector (control) or mGluR7 siRNA and NC-siRNA (control). At 24 hours, 48 hours, and 72 hours after transfection, cell proliferation was detected with MTT and neurosphere diameter was measured. (A) Proliferation of NSCs after transfection with mGluR7 overexpression plasmid. (B) Proliferation of NSCs after transfection with mGluR7 siRNA. (C) Neurosphere diameter after transfection with mGluR7 overexpression plasmid. (D) Neurosphere diameter after transfection with mGluR7 siRNA. (E) Expression of Ki-67 after transfection with mGluR7 overexpression plasmid or mGluR7 siRNA by Western blot. *, compared with the corresponding NC-siRNA (negative control) group or control (empty vector) group, n = 3, $P < .01$. mGluR7 = metabotropic glutamate receptor 7, NSCs = neural stem cells.

the plasmid transfection, demonstrating that the stem cell type is not affected by plasmid transfection (Fig. 1 D)

### *3.2. Effect of mGluR7 on proliferation of human NSCs and neurospheres*

With MTT analysis, we preliminary analyzed the effect of mGluR7 on the proliferation of human NSCs. Compared with the control group, mGluR7 overexpression promoted the proliferation of NSCs while mGluR7 suppression by siRNA inhibited the proliferation of NSCs, at 24 hours, 48 hours, and 72 hours respectively ($P < .01$) (Fig. 2A and Fig. 2B). Then, the diameters of neurospheres were measured. Similarly, the diameters of neurospheres after mGluR7 overexpression were significantly larger, while those after mGluR7 knockdown ($P < .01$) (Fig. 2C and Fig. 2D). The Ki-67 expression was detected with Western blot. The data showed that GluR7 overexpression increased the expression of Ki-67, whereas mGluR7 siRNA decreased the expression of Ki-67 (Fig. 2 E). This data suggests that mGluR7 can promote the proliferation of NSCs.

### *3.3. Effect of mGluR7 on cell cycle of human NSCs*

To determine the effect of mGluR7 on cell cycle, flow cytometry was conducted. As shown in Figure 3A, after overexpression of mGluR7, the ratio of cells in G1/G0 phase decreased and the ratio of cells in S phase increased compared with the control group, suggesting that mGluR7 overexpression promotes the transition of G1 phase to S phase. The difference was statistically significant (Fig. 3B, $P < .01$). Furthermore, compared with the NC-siRNA group, the mGluR7 siRNA group had a significantly higher ratio of G1/G0 phase cells and a significantly decreased S phase cell ratio, indicating that mGluR7 knockdown arrests the cell cycle at G1/G0 phase (Fig. 3C and D, $P < .01$). This indicates that mGluR7 may induce more cells to enter S phase, therefore promoting DNA replication, mitosis of NSCs and the proliferation of NSCs.

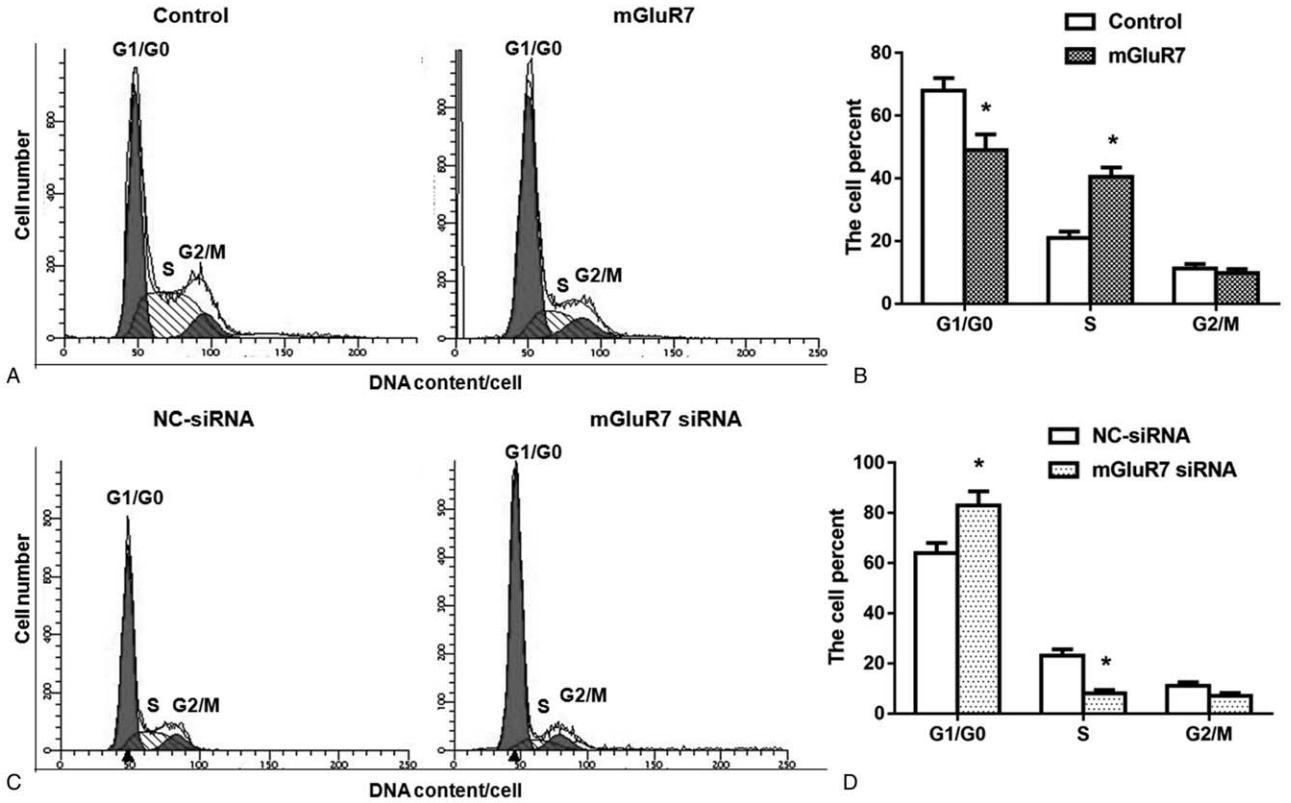


**Figure 3.** Effects of mGluR7 on the cell cycle of human NSCs. NSCs were transfected with pCMV2-GV146-GFP-mGluR7 plasmid and pCMV2-GV146-GFP empty vector (control) or mGluR7 siRNA and NC-siRNA (control). At 24 hours after transfection, cell cycle was detected with flow cytometry. Representative (A) and quantitative flow cytometry results (B) after transfection with mGluR7 overexpression plasmid were shown. Representative (C) and quantitative flow cytometry results (D) after transfection with mGluR7 siRNA were shown. *, compared with the corresponding NC-siRNA (negative control) group or control (empty vector) group, n = 3, $P < .01$. mGluR7 = metabotropic glutamate receptor 7, NSCs = neural stem cells.

### *3.4. Effect of mGluR7 on apoptosis of human NSCs*

The apoptosis of human NSCs was detected by flow cytometry after 48 hours of transfection. It has been found that compared with control group, the percentages of early apoptosis (the lower right quadrant of Fig. 4A) and late apoptosis cells (the upper right quadrant of Fig. 4A) in mGluR7 overexpression group were significantly decreased (Fig. 4B, $P < .01$). On the other hand, transfection of mGluR7 siRNA significantly increased the percentages of the early and late apoptosis of human NSCs (Fig. 4C and Fig. 4D, $P < .01$). These results show that mGluR7 can inhibit the apoptosis of human NSCs.

### *3.5. Effect of mGluR7 on expression of proliferation-rated and apoptosis-related proteins*

To investigate the mechanism of mGluR7 in promoting proliferation and inhibiting apoptosis, we utilized western blots to analyze the expression of key molecules in the human AKT signaling pathway and Caspase-3/9 signaling pathway. After overexpression of mGluR7 in human NSCs, p-AKT was up-regulated, and cyclin D1 was also up-regulated (Fig. 5). These indicate that mGluR7 may up-regulate phosphorylation of the AKT signaling pathway, activate AKT in human embryonic cortical NSCs and further promote the proliferation of NSCs by activating proliferation-associated transcription factors. And mGluR7 promoted the up-regulation of cyclin-dependent kinase CDK2, which may induce more cells to cross G1/S phase nodes, therefore promoting DNA replication, mitosis of NSCs and the proliferation of NSCs. Also, apoptosis-related protein Caspase-3/9 expression was down-regulated, indicating that mGluR7 can inhibit the apoptosis of NSCs. On the contrary, after transfection of mGluR7 siRNA in NSCs, p-AKT, Cyclin D1, and CDK2 were all down-regulated, and caspase-3/9 was up-regulated, suggesting that after mGluR7 knockdown, the proliferation of NSCs is inhibited and cellular apoptosis is induced. Together, these results revel that mGluR7 may promote proliferation of NSCs by up-regulating AKT phosphorylation, cyclin D1, and CDK2, and inhibit apoptosis of NSCs via down-regulating Caspase-3/9.

## 4. Discussion and conclusion

NSCs exist in the subventricular zone of the lateral ventricle and the subgranular zone of the hippocampus dentate gyrus and are normally in a resting, non-activated state.[14] Nerve injury is one of the common severe clinical diseases that endanger human health and cause severe neurological dysfunctions. There is still no effective treatment method. In recent years, the study of NSCs has shed light on the treatment of CNS diseases.[15] It has been reported that neurotransmitter receptors may regulate neurogenesis in adult CNS.[16] Inhibition of metabotropic glutamate type 1 alpha receptor reduced cell proliferation following status epilepticus.[17] Zhang et al found that activation of mGluR5 promoted the proliferation of rat retinal progenitor cell.[18] However, the role and mechanism of mGluR7 in NSC proliferation is not fully understood.

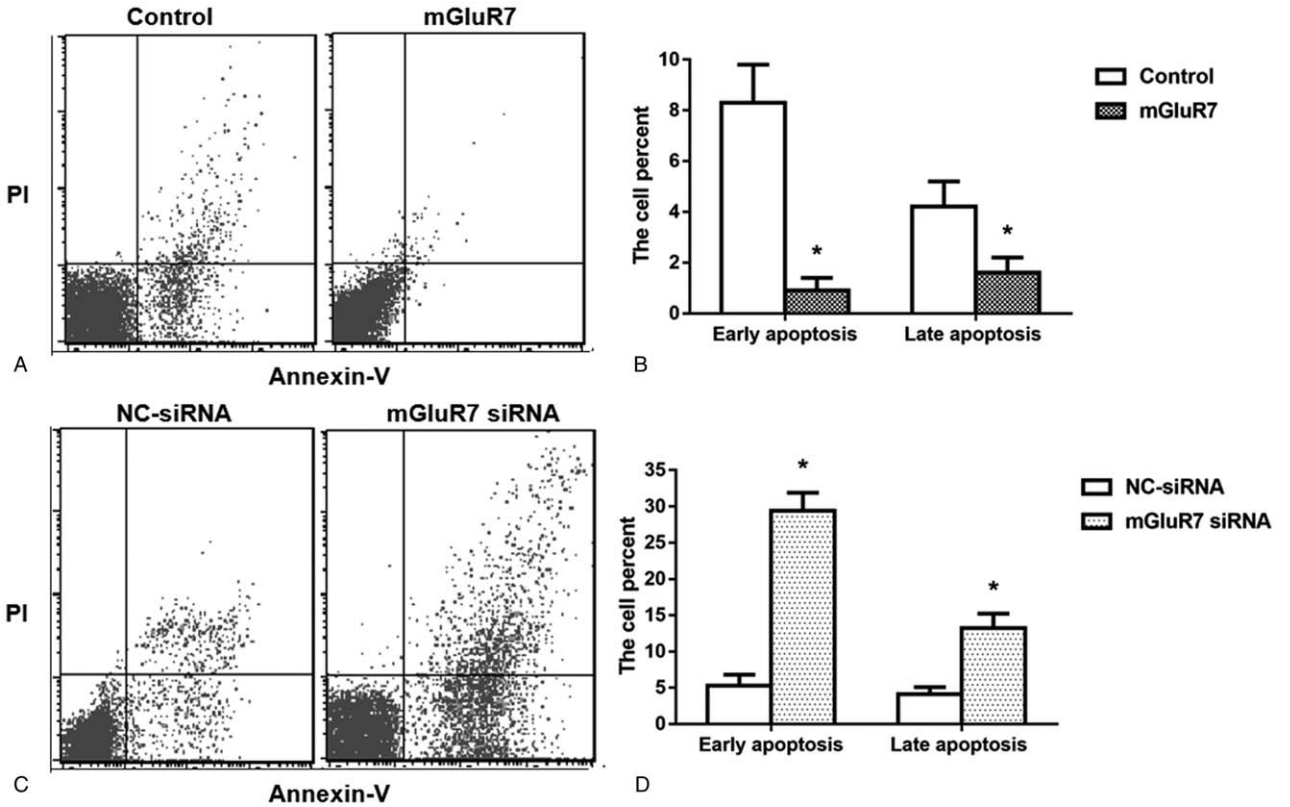


**Figure 4.** Effects of mGluR7 on apoptosis in human NSCs cells. NSCs were transfected with pCMV2-GV146-GFP-mGluR7 plasmid and pCMV2-GV146-GFP empty vector (control) or mGluR7 siRNA and NC-siRNA (control). At 48 hours after transfection, cell apoptosis was detected with flow cytometry. Representative (A) and quantitative flow cytometry results (B) after transfection with mGluR7 overexpression plasmid were shown. Representative (C) and quantitative flow cytometry results (D) after transfection with mGluR7 siRNA were shown. *, compared with the corresponding NC-siRNA (negative control) group or control (empty vector) group, n = 3, $P < .01$. mGluR7 = metabotropic glutamate receptor 7, NSCs = neural stem cells.

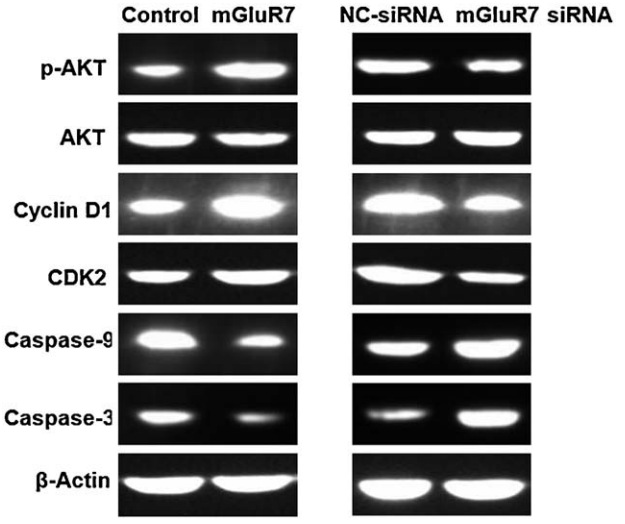


**Figure 5.** Effects of mGluR7 on key proteins of AKT and Caspase-3/9 signaling pathways in human NSCs. NSCs were transfected with pCMV2-GV146-GFP-mGluR7 plasmid and pCMV2-GV146-GFP empty vector (control) or mGluR7 siRNA and NC-siRNA (control). At 48 hours after transfection, protein level was detected with Western blot. Representative Western blot results (A) and quantitative Western blot results (B) were shown. *, compared with the corresponding NC-siRNA (negative control) group or control (empty vector) group, n = 3, $P < .01$. AKT = serine/threonine kinase, mGluR7 = metabotropic glutamate receptor 7, NSCs = neural stem cells.

It is reported that mGluRs (such as mGluR3[19] and mGluR4[20]) play a role in the proliferation of NSCs. Our results found that mGluR7 overexpression enhanced the cell viability and the size of neurospheres of human NSCs cultured in vitro. On the contrary, mGluR7 knockdown by siRNA reduced the cell viability and the size of neurospheres of NSCs. These findings suggest that mGluR7 promotes the proliferation of human embryonic cortical NSCs cultured in vitro.

The G1 phase of the mammalian cell cycle is the only period in which cells respond to environmental signals and thus determine the direction of proliferation, differentiation, or senescence.[21] Important cell cycle regulators, including the Cyclins-CDK4 and CDK6 protein kinase complexes, mediate cell progression through the G1 phase.[22,23] Cyclin D1 and D2 are involved in the development of the nervous system. Cyclin D1 deficiency in rats causes abnormal neurodevelopment and retinal hypoplasia, while Cyclin D2 deficient animals have cerebellar defects.[24,25] Recent studies have shown that Cyclin D1 is important for regulating the proliferation of NSCs during embryonic development, and Cyclin D2 is more important for the adult brain.[26,27]

Cyclin D1 is a key regulator of cell proliferation and cell cycle progression. Several studies[19,20,28] have shown that mGluR1, mGluR3, mGluR4, and mGluR5 can induce cell proliferation by regulating cyclin D1. In this study, the results showed that mGluR7 overexpression promoted cell cycle transition from G1 to S phase whereas mGluR7 knockdown arrested the cell cycle at G1/G0 phase. Also, western blot analysis revealed that the expressions of both cyclin D1 and CDK2 were up-regulated after

overexpression of mGluR7, while they were decreased after mGluR7 knockdown. This suggests that mGluR7 may promote cell cycle progression by up-regulating cyclin D1 and CDK2.

The AKT signal transduction pathway is involved in the regulation of multiple cell functions such as proliferation, differentiation, apoptosis and glucose transport.[29,30] And caspase-3/9 signaling pathway is one of the most classical signal pathways of apoptosis.[31] Both signaling pathways play an important role in the development of the CNS.[32] Tian et al[12] reported that activation of mGluR7 may promote the proliferation and differentiation of neural progenitor cells by influencing phosphorylation of the RAS MAPK signal transduction pathway. AKT is a potential downstream regulator of mGluR7 activation.[33] In recent years, many scholars have been working on the role of phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PI3K)/AKT signaling pathway in the proliferation and differentiation of NSCs. For example, it has been reported that the PI3K/AKT1/mechanistic target of rapamycin kinase (PI3K/AKT1/mTOR) and Ras-ERK signaling pathways are the chief mechanisms regulating the survival, proliferation, and differentiation of NSCs.[34] Omentin can promote the growth and survival of NSCs in vitro through activation of the AKT signaling pathway.[35] Gly-Pro-Glu treatment can promote the proliferation and the migration of NSCs in vitro through a mechanism that involves the activation of ERK and PI3K-AKT pathways.[36] Insulin can stimulate adult NSCs proliferation, but not survival or self-renewal. This effect is mediated by insulin receptor substrate 2 and subsequent activation of the AKT, leading to increased activity of the G1-phase cyclin-dependent kinase 4 and cell cycle progression.[37] Therefore, PI3K/AKT pathways are critical for the proliferation of NSCs. In this study, we found that mGluR7 overexpression promoted the expression of p-AKT levels in human NSCs in vitro, while mGluR7 knockdown reduced it. Therefore, we hypothesize that mGluR7 upregulates AKT phosphorylation in human embryonic cortical NSCs, activates the AKT signaling pathway, and further promotes the proliferation of NSCs by activating proliferation-associated transcription factors. The expression level of apoptosis-related protein Caspase-3/9 can reflect the effect in apoptosis by mGluR7. In this study, after mGluR7 overexpression in human NSCs, the expression of apoptosis-related protein Caspase-3/9 was down-regulated. On the contrary, after the down-regulated expression of mGluR7, the expression of caspase-3/9 increased. This result indicates that mGluR7 can inhibit the apoptosis of NSCs. However, the signaling cascades underlying the effects of mGluR7 seem to be far more complex. Thus, more work is needed to uncover the mechanisms of mGluR7.

Our study demonstrates that mGluR7 promotes the proliferation of human embryonic cortical NSCs in vitro, increases the expression of Cyclin D1 and promotes the expression of cyclin-dependent kinase CDK2 as well as the activation of AKT. In the meantime, the expression of apoptosis-related protein Caspase-3/9 is down-regulated, indicating reduced apoptosis. Our findings may provide experimental evidence that mGluR7 may be used as a potential target for the treatment of focal cerebral ischemic injury and neurodegenerative diseases.

## Author contributions

Jiming Han conceived and supervised the study. Jing Zhang, Junmei Zhao, Yani Chen, Haiyan Shi, Xiaoyong Huang and Yanfeng Wang performed the experiments and collected the data. Jing Zhang and Junmei Zhao prepared the manuscript. Yu Wang and Yameng Wei and Wanjuan Xue performed the statistical analysis. All authors read and approved the final manuscript.

**Conceptualization:** Jiming Han.
**Data curation:** Jing Zhang, Junmei Zhao, Xiaoyong Huang, Yanfeng Wang.
**Formal analysis:** Jing Zhang, Yani Chen, Haiyan Shi, Yameng Wei, Wanjuan Xue.
**Funding acquisition:** Jiming Han.
**Investigation:** Junmei Zhao.
**Methodology:** Yani Chen, Haiyan Shi, Yu Wang.
**Project administration:** Xiaoyong Huang.
**Resources:** Yameng Wei.
**Software:** Jing Zhang, Yani Chen.
**Writing – original draft:** Jing Zhang.
**Writing – review & editing:** Jiming Han.

## References

[1] Guo N, Sahay A. Neural circuits serve as periscopes for NSCs. Cell Stem Cell 2017;21:557–9.
[2] Zhang W, Gu GJ, Zhang Q, et al. NSCs promote hippocampal neurogenesis, metabolic changes and synaptogenesis in APP/PS1 transgenic mice. Hippocampus 2017;27:1250–63.
[3] Lu L, Zhou H, Pan B, et al. c-jun amino-terminal kinase is involved in valproic acid-mediated neuronal differentiation of mouse embryonic NSCs and neurite outgrowth of NSC-derived neurons. Neurochem Res 2017;42:1254–66.
[4] Zhou Y, Wang Y, Olson J, et al. Heparin-binding EGF-like growth factor promotes neuronal nitric oxide synthase expression and protects the enteric nervous system after necrotizing enterocolitis. Pediatr Res 2017;82:490–500.
[5] Ye Q, Wu Y, Wu J, et al. Neural stem cells expressing bFGF reduce brain damage and restore sensorimotor function after neonatal hypoxia-ischemia. Cell Physiol Biochem 2017;45:108–18.
[6] Okazaki H, Kanda A, Kanda S, et al. Cells expressing prominin-1 in neonatal murine inferior colliculus differentiate into neurons and glia. Mol Neurobiol 2018;55:4998–5005.
[7] Qin W, Chen S, Yang S, et al. The effect of traditional Chinese medicine on neural stem cell proliferation and differentiation. Aging Dis 2017;8:792–811.
[8] Chen X, Liu Y, Zhang Z, et al. Hypoxia stimulates the proliferation of rat neural stem cells by regulating the expression of metabotropic glutamate receptors: an in vitro study. Cell Mol Biol (Noisy-le-grand) 2016;62:105–14.
[9] Guerriero RM, Giza CC, Rotenberg A. Glutamate and GABA imbalance following traumatic brain injury. Curr Neurol Neurosci Rep 2015;15:27.
[10] Yang T, Liu YW, Zhao L, et al. Metabotropic glutamate receptor 5 deficiency inhibits neutrophil infiltration after traumatic brain injury in mice. Sci Rep 2017;7:9998.
[11] Erichsen JL, Blaabjerg M, Bogetofte H, et al. Group I metabotropic glutamate receptors: a potential target for regulation of proliferation and differentiation of an immortalized human neural stem cell line. Basic Clin Pharmacol Toxicol 2015;116:329–36.
[12] Tian Y, Liu Y, Chen X, et al. AMN082 promotes the proliferation and differentiation of neural progenitor cells with influence on phosphorylation of MAPK signaling pathways. Neurochem Int 2010;57:8–15.
[13] Jeffries MA, Urbanek K, Torres L, et al. ERK1/2 activation in preexisting oligodendrocytes of adult mice drives new myelin synthesis and enhanced CNS function. J Neurosci 2016;36:9186–200.
[14] Watt FM, Hogan BL. Out of Eden: stem cells and their niches. Science 2000;287:1427–30.
[15] Gonzalez R, Hamblin MH, Lee JP. Neural stem cell transplantation and CNS diseases. CNS Neurol Disord Drug Targets 2016;15:881–6.
[16] Hagg T. Molecular regulation of adult CNS neurogenesis: an integrated view. Trends Neurosci 2005;28:589–95.
[17] Friedman LK, Sharma A, Corcia M, et al. Selective inhibition of metabotropic glutamate type 1 alpha receptor (mGluR1α) reduces cell proliferation and migration following status epilepticus in early development. Int J Dev Neurosci 2016;54:6–21.
[18] Zhang Z, Hu F, Liu Y, et al. Activation of type 5 metabotropic glutamate receptor promotes the proliferation of rat retinal progenitor cell via activation of the PI-3-K and MAPK signaling pathways. Neuroscience 2016;322:138–51.

[19] Guo J, Zhou X, Chen Y, et al. mGluR3 promotes proliferation of human embryonic cortical neural progenitor cells by activating ERK1/2 and JNK2 signaling pathway in vitro. Cell Mol Biol (Noisy-le-grand) 2014; 60:42–9.
[20] Duan Z, Zhang X, Zhu GX, et al. Activation of mGluR4 promotes proliferation of rat neural progenitor cells while mediating activation of ERK1/2 signaling pathway. Cell Mol Biol (Noisy-le-grand) 2013;suppl 59:OL1809–17.
[21] Sherr CJ. G1 phase progression: cycling on cue. Cell 1994;79:551–5.
[22] Sherr CJ. D-type cyclins. Trends Biochem Sci 1995;20:187–90.
[23] Kozar K, Ciemerych MA, Rebel VI, et al. Mouse development and cell proliferation in the absence of D-cyclins. Cell 2004;118:477–91.
[24] Fantl V, Stamp G, Andrews A, et al. Mice lacking Cyclin D1 are small and show defects in eye and mammary gland development. Genes Dev 1995;9:2364–72.
[25] Huard JM, Forster CC, Carter ML, et al. Cerebellar histogenesis is disturbed in mice lacking cyclin D2. Development 1995;126:1927–35.
[26] Sundberg M, Savola S, Hienola A, et al. Glucocorticoid hormones decrease proliferation of embryonic neural stem cells through ubiquitin-mediated degradation of Cyclin D1. J Neurosci 2006;26:5402–10.
[27] Kowalczyk A, Filipkowski RK, Rylski M, et al. The critical role of cyclin D2 in adult neurogenesis. J Cell Biol 2004;167:209–13.
[28] Kanaya S, Komatsu H, Shimauchi H, et al. Metabotropic glutamate receptor 1 promotes cementoblast proliferation via MAP kinase signaling pathways. Connect Tissue Res 2016;57:417–26.
[29] Restuccia DF, Hemmings BA. Cell signaling. Blocking Akt-ivity. Science 2009;325:1083–4.
[30] Wallach D, Kang TB, Dillon CP, et al. Programmed necrosis in inflammation: toward identification of the effector molecules. Science 2016;352:aaf2154.
[31] Srikanth CV, Wall DM, Maldonado-Contreras A, et al. Salmonella pathogenesis and processing of secreted effectors by caspase-3. Science 2010;330:390–3.
[32] Yang SR, Cho SD, Ahn NS, et al. The role of p38 MAP kinase and c-Jun N-terminal protein kinase signaling in the differentiation and apoptosis of immortalized neural stem cells. Mutat Res 2005;579: 47–57.
[33] Yang L, Mao L, Tang Q, et al. A novel Ca2+-independent signaling pathway to extracellular signal regulated protein kinase by coactivation of NMDA receptors and metabotropic glutamate receptor 5 in neurons. J Neurosci 2004;24:10846–57.
[34] Lee JE, Lim MS, Park JH, et al. PTEN promotes dopaminergic neuronal differentiation through regulation of erk-dependent inhibition of S6K signaling in human neural stem cells. Stem Cells Transl Med 2016;5:1319–29.
[35] Zhao LR, Du YJ, Chen L, et al. Omentin-1 promotes the growth of neural stem cells via activation of Akt signaling. Mol Med Rep 2015;11:1859–64.
[36] Almengló C, Devesa P, Devesa J, et al. GPE promotes the proliferation and migration of mouse embryonic neural stem cells and their progeny in vitro. Int J Mol Sci 2017;18:pii: E1280.
[37] Chirivella L, Kirstein M, Ferrón SR, et al. Cyclin-dependent kinase 4 regulates adult neural stem cell proliferation and differentiation in response to insulin. Stem Cells 2017;35:2403–16.