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Expression of Slit2 in neural stem cell differentiation and maturation and its inhibitory effect on axon growth

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The present study was done to investigate Slit2 expression in neural stem cell differentiation and maturation and its inhibitory effect on axon growth. Neural stem cells were extracted from newborn SD rats. After neurospheres were formed, immunofluorescence staining was performed to observe cell morphology and to identify cell types after differentiation and maturation of NSCs. NSCs were then induced by culturing into a differentiation culture medium. Western blotting was performed to detect Slit2 protein expressions. Slit2 siRNA was prepared and transfected into NSCs. Transwell chamber assay was used to observe cell migration and CCK-8 assay was performed to detect cell proliferation. Our results show that NSCs have the potential to differentiate into a variety of neural cells and Slit2 expression is significantly increased during differentiation of NSCs. Slit2 siRNA transfection efficiency showed that Slit2 siRNA was successfully transfected into NSCs, and Slit2 siRNA transfection inhibited NSCs migration and proliferation together with axonal growth of NSCs. Therefore, we conclude that Slit2 can promote NSCs migration and proliferation, and can inhibit nerve cell axon growth, which is favorable for neural network reconstruction.

1. Introduction

Nervous system degenerative or traumatic diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), stroke, etc., are still lacking effective treatments, and various neuroprotectives are also without significant clinical efficacy. At present, endogenous neuronal regeneration and exogenous stem cell transplantation are the two main strategies to promote nerve regeneration and repair. However, the number of endogenous neural stem cells is limited; the survival rate of exogenous neural stem cells after transplantation is low, axon regeneration is difficult, and it is difficult to establish synaptic connections with normal tissues, which are obstacles to stem cell transplantation treatment (Theus et al. 2008). This may be related to the environment around the ischemic focus which is not conducive to the growth of neural stem cells, the cells themselves may express some factors that inhibit their survival and axonal growth.

Previous studies showed that Slits / robos were expressed in both neural stem cells and axons (Kidd et al. 1999), and also expressed in brain and brain lesions in adults. They are involved in the inhibition of nerve and axon regeneration after brain injury formation (Wehrle et al. 2005). Slits, a secreted extracellular matrix protein that acts by binding to its receptor Robos, is a neuro-growth-promoting factor that has a repulsive orientation for both axonal projection and neuronal migration (Andrews et al. 2008; Zhao et al. 2013). In mammals, three Slit (Slit1, Slit2, Slit3) and four of its ligands Robo (ROBO1 ~ ROBO4) have been found (Domyan et al. 2013; Yuen and Robinson 2013; Lucas and Hardin 2017). Slit / Robo signaling pathway can play an important regulatory role in cell migration, inflammatory response, tumorigenesis and organ development by acting as a guide inhibitor of some axons or axon branching and elongation inducing factor (Li et al. 2014; Li et al. 2015). In addition, Slits / Robos expressions can be changed after CNS injury, suggesting that they play a role in adult brain and CNS injury repair (Tonchev and Yamashima 2006; Zhang et al. 2007). Hagino et al. (2013) used *in situ* hybridization to study the expression of Slit mRNA in the brain of mice with cold injury and found that Slits mRNAs were expressed in the lesion area 7 days after injury. Among them, Slit2 mRNA is most expressed in reactive astrocytes around the necrotic area.. Park's results suggest that Slit2 / Robo signaling may be involved in the regulation of astrocytes response through autocrine or paracrine mechanisms after ischemia (Park et al. 2016). Inhibition of Slit2 expression may be beneficial to the formation of ocular neovascularization in mice (Chen et al. 2007). Slit2 may play an important role in the regeneration of neurons and the regeneration of blood vessels. Robo4 acts as a vascular specialized receptor of Slit and may inhibit the regeneration of blood vessels by binding to robo4 (Wang et al. 2007). Nerve regeneration following cerebral ischemia relies heavily on revascularization and is vessel-mediated during its migration to the site of injury (Yamashita et al. 2006; Thored et al. 2007). In conclusion, Slit2 may prevent regenerated axons from entering the lesion and become a cause of CNS aplasia. Therefore, inhibition of the Slits / robos signaling system may promote the axonal regeneration of the peripheral nerve cells in the infarct zone and the migration of endogenous neural stem cells around the infarct zone after brain injury, and may also provide a favorable living environment for the exogenous neural stem cells.

We cultured and identified neural stem cells and investigated the effects of Slit2 siRNA on Slit2 / robos signaling system in proliferation, migration and differentiation of neural stem cells and related mechanisms to provide some theoretical support for neural stem cell transplantation for neurological diseases.

2. Investigations and results

2.1. Identification of NSCs

The neonatal rat hippocampal single cell suspension was inoculated into serum-free medium containing rat bFGF, rat EGF and

B27. By day 2-3, the cells proliferated rapidly, showing clusters of polyclonal balls that formed more than a dozen cells. On the fifth day of culture, the primary neural stem cells, the neurospheres, were basically formed. The edges of the globules gradually became clear. The cells were more refractile and suspended in the medium. There was contact between a few balls (Fig. 1A). On days 7-8, the neurospheres are continuously enlarged to a diameter of about 100-200 μm, at which time subculture can be performed. Nerve balls are digested with accutase for several minutes, and the neural stem cells are then dispersed by mechanical beating. Subculture of neurospheres was also performed using serum-free DMEM / F12 medium containing rat bFGF, rat EGF and B27. Neurospheres were immunofluorescently stained with nestin antibody. As shown in Fig. 1-B, almost all of the third generation neurospheres showed a homogeneous red fluorescence and was positive for nestin staining, indicating that they were neural stem cells.

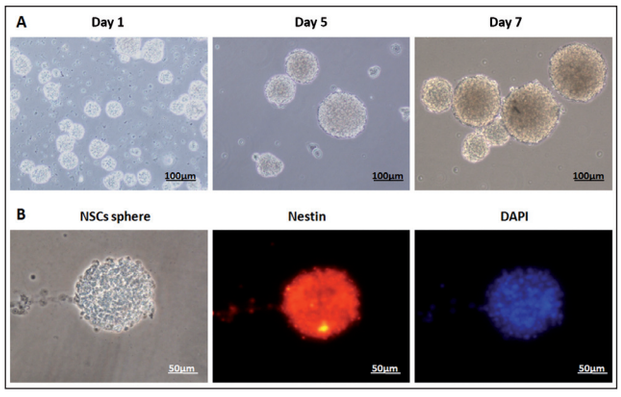

Fig. 1 A: Primary neural stem cell culture (×100); B: immunofluorescence staining of neural stem cells (×200)

2.2. NSCs have the potential to differentiate into a variety of neural cells

The NSCs on day 3 were collected and transferred to DMEM / F12 medium containing 2% fetal bovine serum and 2% B27 to induce differentiation. Fourteen days after induction of differentiation, immunofluorescence staining of cells was performed using GFAP (labeled astrocyte) and β-tubulin III (labeled neuron). The results showed a large number of GFAP-positive cells (Fig. 2), such cells were star-shaped and had large cell body, there are more different lengths, uneven diameter bulge, from the original boundary of the neurospheres to the outermost edge of differentiation, and a dense network of cells is formed. The number of β-tubulin III positive cells is relatively small, and the cell body is smaller, mostly round, protruding one or a few small and long protrusions (Fig. 2). β-Tubulin III-positive cells either alone or in small numbers, scattered between GFAP-positive cells. The experimental results show that neural stem cells isolated from the hippocampus of neonatal rats have the potential to differentiate into different types of neural cells. This multidirectional differentiation potential offers the possibility of stem cell therapy.

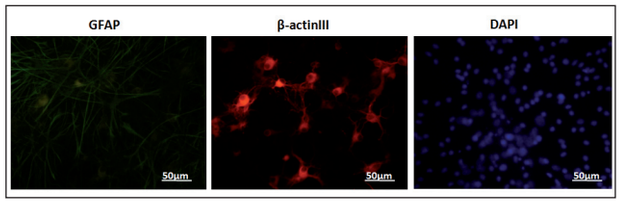

Fig. 2: Immunofluorescence staining of NSCs at 14 days of differentiation showed that green fluorescence was GFAP positive cells, that is, astrocytes (×200); The red fluorescence is β-tubulin III positive cells, namely neurons (×200); the blue fluorescence is the stained nucleus.

2.3. The expression of Slit2 was significantly increased during the differentiation of NSCs

The expression of Slit2 and robo1 during neural stem cell differentiation was detected by Western blot to investigate whether Slit2 / robo1 affects the differentiation of neural stem cells by regulating their expressions. The results showed that with the extension of cell differentiation time (1d-10d), various types of neurons gradually matured. During this process, Slit2 expression showed a significant upward trend (Fig. 3B, P <0.01). Slit2 expression was significantly increased on day 7 and day 10 compared to day 1 (Fig. 3A); meanwhile, robo1 expression also increased slightly (Fig. 3B), but was significantly lower than that of Slit2. It is suggested that the Slit2 / robo1 signaling pathway may promote the migration of neural stem cells out of the neurospheres by increasing the expression of Slit2 protein during differentiation of NSCs, and differentiate them into cell types such as astrocytes and neurons.

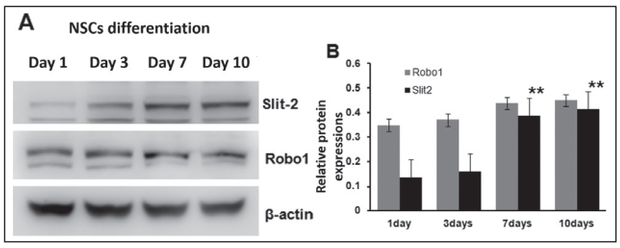

Fig. 3: Slit2 and Robo1 expression at different times of differentiation. A: Western blot results; B: Relative protein expressions of Slit2 and Robo1, ** indicated that $P < 0.05$ compared to day 1.

2.4. Transfection efficiency of Slit2 siRNA

In order to detect the transfection efficiency of siRNA cells, siRNA with green fluorescence was used as a negative control group. SiRNA transfection was performed on the next day after passage of neural stem cells. After transfection for 24-48 h, the cells were observed under a fluorescence microscope and photographed. Most of the stem cells show green fluorescence, indicating that most cells have been successfully transfected with siRNA (Fig. 4). However, larger neurospheres that were not blown off during passage were difficult to successfully transfect (black and white arrows in Fig. 4), maybe because neurospheres are large and siRNAs have difficulty accessing their internal cells. Western blot method was used to detect the interference effect of siRNA at the protein level. As shown in Fig. 5, Slit2 siRNAs at both 50 nM and 100 nM significantly reduced Slit2 protein expression (p <0.01) after 48 h of interference as compared to the negative control group. Among them, Slit2 siRNA 100 nM had the best interference effect, but it still could not completely inhibit intracellular Slit2 expression. According to the results, we chose 100 nM of siRNA to interfere with Slit2 for follow-up experiments.

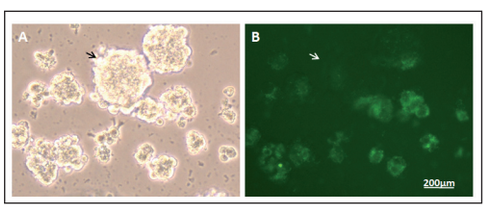

Fig. 4: NSCs transfection results (×200). Unblown cell spheres were found to be difficult to transfect (blank and white arrows in the figure); other cells carrying green fluorescence indicated successful transfection.

2.5. Slit2 promotes NSCs migration

We added blank control, negative control, Slit2 factor and Slit2 siRNA-treated cells to the upper chamber of transwell chamber to observe the migration of neural stem cells into the lower chamber

under different conditions. As shown in Fig. 6-C, more stem cells migrated to the lower chamber in Slit2 factor groups compared to the blank control group, and the number of migrating cells was significantly different (p <0.01). There was no significant difference (p = 1.00) in the number of neural stem cells migrating into the lower compartment between negative control group and Slit2 siRNA group. The above results indicated that the Slit2 factor at a certain concentration had the effect of promoting the migration of neural stem cells, the effect was dose dependent, and inhibited Slit2 had no significant effect on the migration of neural stem cells.

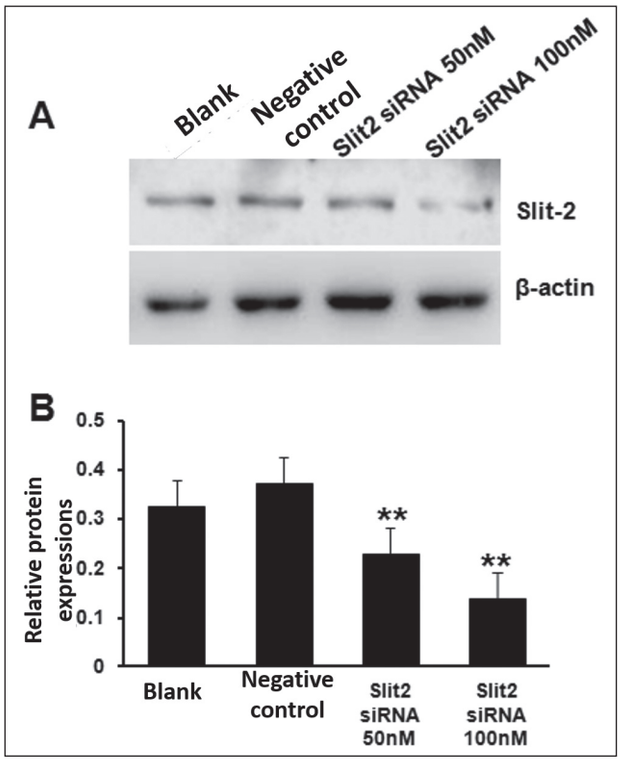

Fig. 5 A: Western blot results after 48h of Slit2 siRNA intervention; B: Relative protein expression of Slit2, ** indicated that $P < 0.05$ compared to Blank and negative control group

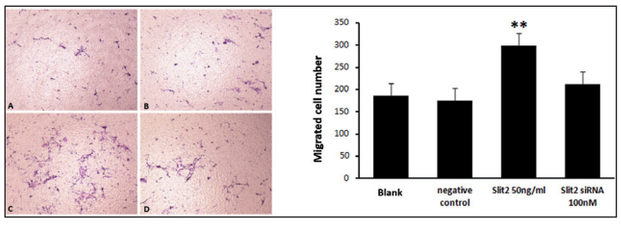

Fig. 6: Slit2 promoted NSCs migration. A: Blank; B: Negative control; C: Slit2 50 ng/ml; D: Slit2 siRNA. ** indicated that $P < 0.05$ compared to Blank and negative control group

2.6. *Slit2 promotes NSCs proliferation*

CCK-8 result is shown in Fig. 7. Compared with the blank control group, Slit2 10 ng/mL and 50 ng/mL significantly increased cell proliferation (P = 0.023 and 0.024), while Slit2 100 ng/mL treatment had no significant difference (p = 0.153), probably because of the high concentration of Slit2 factor has some toxic effects on cells, and this concentration cannot promote its proliferation. NSCs proliferation showed no significant difference between Slit2 siRNA group and negative control group. From the above results, the appropriate Slit2 factor may be beneficial to promote the proliferation of neural stem cell, help to increase the number of stem cells, but high concentrations of Slit2 factor was not conducive to the proliferation of neural stem cells.

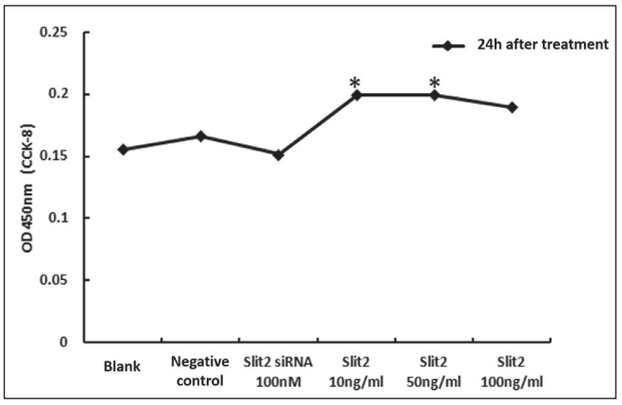

Fig. 7: Slit2 promoted NSCs proliferation. * indicated that p <0.05 compared to blank group.

2.7. *Slit2 inhibits axonal growth of NSCs*

We added different concentrations of the Slit2 factor in the stem cell culture medium and found that all the concentrations were effective in inhibiting the axonal growth of adherent neural stem cells compared with the control group (Fig. 8A) and significantly reduced adhesion (Fig. 8 D, E, F). The higher the concentration of Slit2 the better is the inhibitory effect, of which the 50 ng/mL Slit2 effect is particularly significant (Fig. 8E), the figure shows that most of the neural stem cell axon growth was inhibited. The results showed that neural stem cells in the 10 ng/mL Slit2-treated group were larger than those in the control group. Compared with the 10 ng/mL group, the volume of neurospheres was slightly smaller in the 50 ng/mL group and the 100 ng/mL group, and the higher concentration of Slit2 may partially inhibit the proliferation of stem cells. It was shown that the effect of Slit2 on neuronal axon inhibition is also valid for undifferentiated neural stem cells. Therefore, adding an appropriate amount of Slit2 during the process of neural stem cell culturing may effectively prevent the adherent process during passage and increase the number of neural stem cells. In contrast, inhibition of Slit2 may increase the intrinsic ability of axons to grow and regenerate, favoring neural network reconstruction and restoration of cellular function. In the Slit2 siRNA group, neural stem cells had longer axons after adherent, compared to negative control. Therefor it can be seen that Slit2 inhibition is conducive to axonal growth.

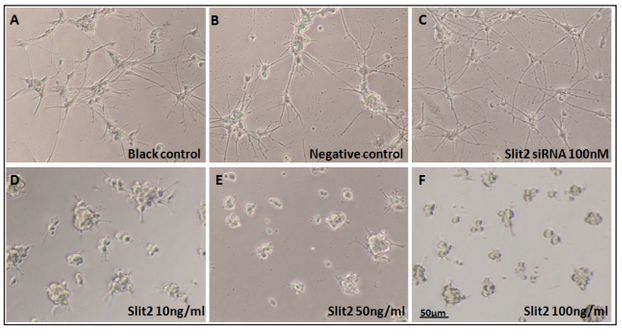

Fig. 8: Slit2 inhibited NSCs axon growth (×100). A: Blank; B: Negative control; C: Slit2 siRNA 100 nM; D: Slit2 10 ng/mL; E: Slit2 50 ng/mL; F: Slit2 100 ng/mL

3. Discussion

Early studies of the Slit/Robo signaling pathway focused on the nervous system and found that Robo can excite neuronal axons repeatedly across the neural tube midline during neurite outgrowth and neural cell migration, with the deepening of research, it was found that it also has the role of promoting axon branching and regulating neuronal migration.

As a kind of exogenous cell replacement therapy, neural stem cell transplantation is considered as the most potential new method to treat cerebral ischemia, which is a hot research topic of ischemic cerebrovascular disease in recent years (Martinez et al. 2018). Poor survival after neural stem cell transplantation, axonal regeneration and the establishment of synaptic connections with normal tissue are obstacles in stem cell transplantation (Li et al. 2017). This is mainly related to the environment around the ischemic foci after stem cell transplantation which is not conducive to its growth and the stem cells themselves may express some factors that inhibit their survival and axonal growth. Slits / robos research has focused on the development of the nervous system, which is expressed on neural stem cells and on axons (Bhat 2017). Recent studies have found that they are expressed in adult brain and around brain lesions and participate in prevention of neurogenesis and axonal regeneration after brain injury (Lei et al. 2017). Therefore, Slit2 siRNA was applied to the treatment of neural stem cells to inhibit the signal pathways of Slit2 / robo and to study their effects on the proliferation and migration of neural stem cells in order to provide theoretical support for the treatment of exogenous neural stem cells.

The regenerative process of nerve injury often repeats many of the features of the developmental stage. The findings suggested that normal development and regeneration of the nervous system depended on two mechanisms: the accurate projection of axons and the neuronal migration. In recent years, studies have found that certain traditional Chinese medicines, growth factors and small molecule compounds have the function of regulating the proliferation and differentiation of stem cells *in vivo*, while the degenerative or traumatic diseases of the nervous system including Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), depression and stroke are all diseases caused by the loss or damage of nerve cells. Drugs are used to stimulate and regulate the proliferation and differentiation of neural stem cells in order to reconstruct damaged functional cells and restore their biological functions. It not only solves the problem of the origin of adult neural stem cells and the ethical confusion of embryonic stem cells but also avoids the problem of immunological rejection and surgical sequelae of cell transplantation. This study provides a new perspective and ideas for the prevention and treatment of cell damage diseases.

Brain blood vessels, nerve cells and matrix signaling between the dynamics of the extracellular matrix microenvironment play an important role in survival of nerve cells, maintaining the neurovascular units of brain cells in a stable state is the basic of all normal functions of brain cells. Cerebral ischemia in the microenvironment will have a huge change, produce a variety of cytokines, on the one hand induce a large number of lateral ventricle of neural progenitor cells proliferation and migration to the damaged parts of differentiation, on the other hand induce a large number of vascular endothelial cell proliferation and promote blood vessels regeneration, the two complement each other, simultaneously (Palmer et al. 2000; Wei et al. 2001).

In addition, recent studies have found that nerve regeneration following cerebral ischemia relies heavily on revascularization, especially on vascular remodeling during its migration to the damaged site (Yamashita et al. 2006; Thored et al. 2007). Inhibition of vascular regeneration after cerebral ischemia can inhibit migration and differentiation of nerve cells and thus inhibit nerve regeneration. It is more reasonable to treat cerebral ischemia by promoting regeneration of blood vessels and nerves and improving microenvironment after cerebral ischemia.

In this study, we investigated the effects of Slit2 on the differentiation and maturation of neural stem cells and its effect on the migration and proliferation of NSCs by cell culture, immunofluorescence, cell transfection, Western blot and cell migration assays, and the effects of Slit2 on axonal growth during subculturing NSCs, following results and conclusions are obtained: (1) neural stem cells exist in neonatal rat hippocampus, which can be extracted and cultured *in vitro*, after continuous passage, higher purity of neural stem cells were obtained. *In vitro* cultured neural stem cells have been identified with multi-directional differentiation potential. (2) With the extension of neural stem cell differentiation, many types of neural cells tended to mature, and Slit2 expression showed a significant upward trend; studies have shown that Slit2 factor has the effect of promoting neural stem cell migration, proper addition of Slit2 may be beneficial in promoting the proliferation of cultured neural stem cells. Therefore, the Slit2 / Robo1 signaling pathway may promote neural stem cells migrating out of neurospheres and differentiating into astrocytes and neurons by increasing Slit2 expression. (3) Experiments show that the inhibitory effect of Slit2 on nerve cell axon also applies to adherent neural stem cells. Addition of appropriate amounts of Slit2 in the process of neural stem cell culture can effectively prevent the adherent process during passage and increase the number of neural stem cells. Inhibition of Slit2 may enhance the intrinsic growth and regeneration of axons, which is good for neural network reconstruction.

4. Experimental

4.1. Primary culture and passage of NSCs

Take five newborn SD rats (within 24 h), 75% ethanol for 1 min × 2 times, transferred to PBS for 2 times, quickly placed on ice after breaking head. Cut the scalp and skull layer by layer with the eye scissors from the midline of the occipital part of the skull. After fully exposing the brain, remove the intact brain with a spoon and wash twice in precooled PBS. The brain was transferred to a petri dish containing prechilled PBS, the meninges were dissected under a dissecting microscope, the left and right hemispheres were separated from the midline of the brain, the hippocampus was removed, placed in prechilled DMEM / F12 medium, and rapidly sheared, repeatedly blow with a straw to form a single cell suspension; 200 mesh sieve was used to remove uncut tissue block, centrifuged at 1000 rpm for 5 min, the supernatant was discarded, and 2 mL NSCs complete medium was added (DMEM / F12 containing 2 % B27, 20 ng/mL bFGF, 20 ng/mL EGF, and 1% penicillin + streptomycin). Resuspend the pellet, take 10 µl cell suspension for trypan blue staining, after cell count, cells with 5×10^5/mL concentration were inoculated into 25 cm^2 flask, cultured in 37 °C, 5% CO_2 incubator. After being cultured for 6 to 7 days, primary neural stem cells can form a large number of neurospheres in suspension. When neurosphere diameter reached 150-200 µm, cell passage can be done.

4.2. Immunofluorescence staining

The neurospheres (2-3 days after passage) were selected and collected and seeded into 12-well plates with sterile coverslips (polylysine coated) in a 37 °C, 5% CO_2 incubator for 4 h. The cells were aspirated and washed with PBS for 5 min × 3 times and 4% paraformaldehyde for 30 min. The cells were washed with PBS for 5 min × 3 times and incubated with 1 mL / L Triton/PBS for 10 min at room temperature. The cells were rinsed with PBS for 5 min × 3, BSA blocking for 1 h at room temperature. Anti-rat nestin polyclonal antibody (1: 100) was added and incubated overnight at 4 °C. PBS rinse 5 min × 3 times, add the appropriate secondary antibody (goat anti-rabbit FITC, 1: 100), and incubated at room temperature for 1.5 h in the dark, PBS rinsed 5 min × 3 times. DAPI counterstained nuclei for 10 min, rinsed in PBS for 5 min × 3 times and blocked with 90% glycerol. Replaced the primary antibody with PBS in control group, the cells were observed and photographed under an inverted fluorescence microscope.

4.3. Identification of cell types after differentiation and maturation of NSCs

Neurospheres cultured for 3-5 days were seeded at a cell density of 5×10^3 cells / mL in 24-well plates pre-treated with cover slips treated with PDL, add 2% FBS, cultured in 37 °C, 5% CO_2 incubator, induced for 14 days. Aspirate the culture medium, wash it with PBS buffer for 3 times, fix it with 4% paraformaldehyde for 30 min, rinse it with PBS/PBS for 10 min with 1 ml/L triton/PBS for 10 min, mouse anti-β-tubulin III (1: 200), rabbit anti-GFAP (1: 400) were applied, wet box overnight at 4 °C. Rinse 3 times with PBS buffer for 5 min each time. Goat anti-rabbit IgG-FITC (1: 100), goat anti-mouse IgG-Cy3 were applied, dark at room temperature for 2 h, PBS buffer rinse 3 times, each time 5 min. DAPI counterstained nuclei for 10 min. Buffer glycerol packing. Observe and take photos under a fluorescence microscope.

4.4. Induction and differentiation of NSCs

The third generation of NSCs were collected and centrifuged at 1000 r/min for 5 min. After the supernatant was discarded, 1 mL of Accutase was added, and digested at 37 °C for 10 min. The cells were suspended in 5 mL of DMEM / F12 medium and centrifuged at 1000 r/min for 5 min. After repeatedly blow with a pipette to disperse the neurospheres into individual cells, the cells were counted in a hemocytometer and seeded into 24-well plates previously treated with PDL at a cell density of 2×10^5 / mL, and were randomly divided into 1 d, 3 d, 7 d and 10 d groups, cells were cultured in a 5% CO_2 incubator at 37 °C for 1 d, 3 d, 7 d and 10 d respectively. Induction differentiation medium is a primary medium without growth factor. Add 2% FBS and change the medium once every 3 days.

4.5. Western Blotting

A bicinchoninic acid (BCA) kit (Sigma-Aldrich, Customer Support PO Box 14508 St. Louis, MO 63178 USA) was used to detect the protein concentration. After sample

buffer was added to the proteins (each well, 30 μg per sample), proteins were boiled at 95 °C for 10 min. Then, the proteins were separated using 10% polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes with 100 V transfer-molded voltage lasting for 45 to 70 min. Then, samples were incubated at room temperature for 1 h with 5% bovine serum albumin (BSA), and were incubated with primary antibodies (1: 1000 dilution), at 4 °C overnight. After that, samples were washed with tris-buffered saline Tween 20 (TBST) 3 times (5 min/time). The corresponding secondary antibody was added for incubation at room temperature for 1 h. After which membranes were washed 3 times (5 min/time). Development was completed with chemiluminescence reagents. β-Actin was used as an internal reference. Bands were visualized with a Bio-Rad Gel Doc EZ imager (Life Science Research, Education, Process Separations, Food Science, 2000 Alfred Nobel Drive, Hercules, California 94547, USA). ImageJ software (National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland 20892) was applied to analyze the intensity of the target bands.

4.6. Cell transfection

On the day of transfection, 100 μL cell culture medium containing 2×10^5 cells was inoculated into each well of a 24-well plate. First, add 400 μL of nuclease water to the tube, shake for 10 s, and dissolve the fat. Add a certain volume of serum-free medium in a transfection tube, appropriate quality siRNA, and a suitable volume of transfection reagent HiPerFect Reagent after shaking. siRNA negative control sequence: 5'-UUCUCCGAACGUGUCACGUTT-3 '; Slit2 siRNA Target Sequences: 5'-CTGTATCAGTAATCCATGTAA-3' and 5'-ATCAATATTGATGATTGCGAA-3 '. The mixture was allowed to stand at room temperature for 10-15 min to fully form the complex. The mixture was added to the cells, gently mixed and then placed in the incubator and cultured for 6 h. After arrival, complete medium was added for 24-48 hours of culture. 24-48 h after transfection, the experimental results were observed and the expression of green fluorescent protein was recorded, and the interference effect was detected by Western blot.

4.7. Transwell chamber assay for cell migration detection

After termination of digestion, the medium was removed by centrifugation, washed with 0.01 M PBS 1-2 times and resuspended in NSCs complete medium. Adjust the cell density to $1.0\text{-}1.6 \times 10^5$ cells / mL. Take 100 μL of cell suspension under different processing conditions into Transwell chamber (Corning) and add 600 μL of medium containing FBS in 24-well plate and incubate for 12-24 h. Wipe the cells in the upper chamber with a cotton swab, stain with 0.1% crystal violet for 5 min, and wash with 0.01M PBS three times. Use Nikon inverted microscope for observation and photographing, randomly select a number of visual field counting cells.

4.8. CCK-8 assay for cell proliferation detection

Cell suspension (100 μl) was seeded in a 96-well plate at a density of 1×10^4 cells / well and cultured at 37 °C in a 5% CO_2 incubator for 24 h. Add 10 μL of different concentrations of the test substance into the culture plate. Incubate for the appropriate time (6, 12, 24, or 48 h). Add 10 μL of CCK-8 solution to each well. Incubate plates for 1 to 4 h. Before reading the plate, gently shake the plate for 1 min to ensure uniform color distribution. Measure the absorbance at 450 nm with a microplate reader.

4.9. Cell grouping

The third generation of neural stem cells with good growth condition were selected and centrifuged at 1000 r/min for 5 min, and the supernatant was discarded. Accutase 1 mL was added and 37 °C for 10 min, then added to 5 mL DMEM / F12 medium and centrifuged for 5 min at 1000 r/min. Add 500 μL of NSCs complete medium (without penicillin - streptomycin), gently blow to make the neurospheres dispersed into single cells, after the cells were counted, cells were inoculated into petri dishes with a concentration of 5×10^5 / mL, cultured for 12 h, randomly divided into the following groups: (1) control group: without transfection or addition slits2; (2) negative control group: transfected with negative siRNA which has no effect of inhibition; (3) Slit2 siRNA transfection group: transfected with siRNA 100 nM; (4) Slit2 10 ng/mL; (5) Slit2 50 ng/mL; (6) Slit2 100 ng/mL. The above treatment groups were cultured at 37 °C in a 5% CO_2 incubator for 24 h, photographed under an inverted microscope and the results recorded.

4.10. Statistical analysis

All data were analyzed by GraphPad Prism version 6 statistical software. Measurement data were expressed by mean±standard deviation. The t test was used for comparisons between two groups after confirming Gaussian distribution of the data. One-way analysis of variance was applied for comparisons between multiple groups. Statistical significance was assumed for $P < 0.05$.

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Conflicts of interest: None declared.

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