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The Journal of Biomedical Research, 2022 36(2): 127–140

JBR

Original Article

Valproic acid enhances neurosphere formation in cultured rat embryonic cortical cells through TGFβ1 signaling

Cui Qi[1,△], Jiaqi Zhang[1,△], Yuanyuan Wang[1], Mingyan Lin[1], Jun Gao[1,2,✉], Haiying Lu[3,✉]

[1]Department of Neurobiology, Key Laboratory of Human Functional Genomics of Jiangsu, School of Basic Medical Sciences, Nanjing Medical University, Nanjing, Jiangsu 211166, China;
[2]Department of Rehabilitation Medicine, Jiangsu Shengze Hospital affiliated to Nanjing Medical University, Suzhou, Jiangsu 215228,China;
[3]Department of Neurology, Children's Hospital of Nanjing Medical University, Nanjing, Jiangsu 210008, China.

Abstract

This study aimed to investigate the effect and mechanism of valproic acid (VPA) on the neurosphere formation in rat embryonic cortical cells. We used free-floating neurosphere formation as a model system to evaluate the VPA on the proliferation of neural stem cells (NSCs). We found a time- and dose-dependent increase in neurosphere formation and NSC proliferation after VPA treatment. Further RNA-seq analysis demonstrated that the upregulated TGFβ1 signaling might attribute to the effect of VPA on the neurosphere formation and NSC proliferation. Consistently, the neurosphere formation and NSC proliferation were blocked by the treatment with SB431542, an inhibitor of TGFβ1 receptor. Moreover, in a coculture system, NSCs treated with VPA significantly reduced the oxygen-glucose deprivation-induced neuronal apoptosis. Taken together, our results showed that VPA could enhance neurosphere formation and NSC proliferation by activating TGFβ1, which might be a novel therapeutic strategy for neurological disorders.

Keywords: valproic acid, neural stem cells, neurosphere formation, proliferation, transforming growth factor β1

Introduction

Neural stem cells (NSCs), a class of stem cells with self-renewal ability, have the potential to differentiate into neurons, astrocytes, and oligodendrocytes. The NSCs are proposed to be used in the treatment of Huntington's disease, stroke, Alzheimer's disease, and other psychiatric and neurodegenerative diseases[1]. Though widely studied, there is still much work to do before the applications of NSCs in clinical practice. A stable and reliable source of NSCs has always been a challenge. Since differentiation and self-renewal can both be evaluated at the single cell level theoretically, the sphere forming assays are extensively used in stem cell biology. Thus, besides the quantitative readout of the stem cell number *in vivo*, the self-renewal and differentiation and functional properties of the stem cells can all be exhibited by the neurosphere assay[2].

△These authors contributed equally to this work.

✉Corresponding authors: Jun Gao, Department of Neurobiology, School of Basic Medical Sciences, Nanjing Medical University, 101 Longmian Avenue, Jiangning District, Nanjing, Jiangsu 211166, China. Tel: +86-25-86869347, E-mail: gaojun@njmu.edu.cn; Haiying Lu, Department of Neurology, Children's Hospital of Nanjing Medical University, 72 Guangzhou Road, Gulou District, Nanjing, Jiangsu 210008, China. Tel: +86-25-83116827, E-mail: hylunj@163.com.

Received: 16 July 2021; Revised: 30 November 2021; Accepted: 07 December 2021; Published online: 15 February 2022

CLC number: R741, Document code: A

The authors reported no conflict of interests.

https://doi.org/10.7555/JBR.36.20210109

It is believed that promoting neurosphere formation of NSCs *in vitro* is a relatively simple and efficient pathway to obtain more NSCs.

The differentiation and self-renewal of the NSCs have been shown to be closely associated with the epigenetic modulation according to the recent progress in the field[3]. In controlling the fate determination, self-renewal and pluripotency of stem cells, histone deacetylases (HDACs) have been reported to be coordinate with various signaling pathways and cell-intrinsic transcription factors[4]. In embryonic stem cells HDAC1 has been revealed by genetic studies to be a major deacetylase. Proliferation defects have been noticed in the HDAC1-null embryonic stem cells[5]. Moreover, increased NPC proliferation resulted in failed differentiation into mature neurons by deletion of HDAC1 and HDAC2 in the CNS[6]. Besides, both HDAC3 and HDAC5 coordinate with the transcription factor TLX to control NSC self-renewal and proliferation[7].

In the treatment of diverse neurodegenerative diseases, HDAC inhibitors could become novel therapeutic tools in view of the significant role of the HDACs in the control of NSC neurogenesis and proliferation[8]. Studied widely in various tissues and cell types along with the neural progenitor cells (NPCs), valproic acid (VPA) has been known as a HDAC inhibitor. The reduced adherent growth of NPC proliferation can be indicated by VPA[9]. In the promotion of the neuronal growth in cortical cells isolated from E18 embryos cultured *in vitro*, VPA can perform the same function as neurotrophic factors[10]. In the embryonic hindbrain, earlier studies have indicated a proliferative effect of VPA on NSCs. Contrarily, in the optic tectum of adult zebrafish, VPA decreases cell proliferation[11]. VPA can also inhibit neurosphere formation in the adult subventricular zone[12]. However, the effect of VPA on free-floating neurosphere formation in the embryonic cortical cells cultured *in vitro* is not fully understood.

In this study, we investigated the effect and mechanism of VPA on the neurosphere formation in cultured rat NSCs. By morphological observation and CCK8 assay, we observed that VPA treatment promoted the neurosphere formation of NSCs. Moreover, the results from RNA-seq and real-time quantitative PCR suggested that the TGFβ1 signaling pathway may be involved in this process. Furthermore, the co-culture of VPA-treated NSCs with neurons could significantly reduce the cell death induced by oxygen-glucose deprivation (OGD), which suggests a potential application of VPA in clinical practice.

Materials and methods

Cell culture

NSC culture

NSCs were isolated from the embryonic cortical tissues of Sprague-Dawley (SD) rats (Nanjing Medical University) at 12.5–14.5 days of pregnancy. Isolated fetal rat brain tissue was immersed in Hanks' balanced salt solution, and the tissue fragments were gently triturated using a 2 mL pasteur pipette to produce a suspension of single cells. The resulting cells were centrifuged for 3 minutes at 500 *g*; the supernatant was then aspirated and discarded. Then, NSCs were expanded as neurospheres in DMEM/F12 (Gibco, USA), which was supplemented with 2% B27 (Gibco), 20 ng/mL epidermal growth factor (EGF) (Gibco) and 20 ng/mL basic fibroblast growth factor (bFGF) (Gibco). Cell viability was assessed with trypan blue (Sigma, USA), and NSCs were plated in 10 cm Petri dishes at a density of 2×10^5 cells/mL. NSCs were then incubated at 37 °C in a 5% CO_2 incubator (Thermo, USA). The medium was changed every other day by removing half of the medium and adding fresh growth medium. After 5 to 7 days, the NSCs were passaged by TrypLE Express (Gibco). The NSCs were used to study the neurosphere formation.

Hippocampus neuron culture

Based on the earlier reports the primary culture of rat hippocampus neurons was conducted. The SD rats were supplied by the Animal Centre of Nanjing Medical University. All the procedures adhered to the national standards. Subsequent to trypsinization of 4 mL of 0.25% trypsin (Gibco) for 2 minutes at 37 °C, the hippocampus from the fetal rats on embryonic 18 days was interrupted using a 0.5 mL fetal bovine serum. By centrifugation at 900 *g* for 10 minutes, the cells were collected. Using the minimum essential medium Neurobasal (Gibco), the pellet was resuspended. On a pretreated with 100 μg/mL Poly-D-Lysine (Sigma) 22 mm × 22 mm glass cover slip the 1×10^6 cells were plated. Immediately before use, 2% B-27 supplement and the 2 mmol/L glutamine (Sigma) were added to the Neurobasal. With 500 μL culture medium, incubation of the 24 well plates was done with 5% CO_2/95% O_2 incubator at 37 °C. Every 3 days, half of the culture medium was changed. On day 10 all the *in vitro* experiments were conducted to set up the OGD-injured model.

The animal study protocol was reviewed and approved by the Ethics Committee of Nanjing Medical University.

Drug treatment

VPA (Sigma) was dissolved in water and stored in a stock solution of 1 mol/L or 10^{-3} mol/L at −20 °C. Then it was diluted to the final concentrations (1, 50, 100, 500, 1000, and 10 000 μmol/L) in the standard external solution before treatment.

SB431542 (Selleck, USA), the inhibitor of TGFβ1 receptor kinase activity, was dissolved in DMSO and stored in a stock solution of 10 mmol/L at −20 °C. SB431542 at the final concentrations of 1 μmol/L and 5 μmol/L was used to treat NSCs with or without 100 μmol/L VPA for 48 hours, respectively, before the effect of VPA on NSCs was observed.

Immunocytochemical staining

To identify NSCs, the immunocytochemical staining was performed. Using 4% paraformaldehyde, the NSCs were fixed at room temperature for 1 hour prior to three times of washing with phosphate buffered saline (PBS) for 5 minutes. Subsequently, for 10 minutes, the cells were permeabilized with 0.2% Triton X-100 through incubation. To block the nonspecific binding, the cells were treated at 37 °C for one hour with 5% bovine serum albumin. The cells were incubated with primary antibodies against SOX-2 or Nestin (Millipore, USA; dilution 1:400) overnight at 4 °C, followed by incubation with secondary antibodies, *i.e.,* Cy3-conjugated anti-rat IgG (Proteintech, China; dilution 1:500) against SOX-2 or FITC-conjugated goat anti-mouse IgG (Bioworld, China; dilution 1:500) against Nestin for 1 hour. The cells were then rinsed for 10 minutes and incubated with PBS thrice in the dark. Nuclei were stained with DAPI. An inverted fluorescence microscope was used to identify NSCs.

Cell viability and cell proliferation assay

Following the instructions of the manufacturer, the Cell Counting Kit-8 (Donjindo, Japan) was used to detect the cell viability. NSCs were seeded in 96-well plates at a density of 3×10^4 cells/well in triplicate and subjected to different treatments. NSCs were treated with VPA at a concentration of 100 μmol/L and at different times (0, 24, 48, and 72 hours). Ten microliters of CCK8 solution were added to each well (containing 100 μL medium), and cells were cultured for an additional 30 minutes at 37 °C. The absorbance of each group at 450 nm was measured using an absorbance microplate reader (Tecan, Switzerland).

The lactate dehydrogenase (LDH) was identified as a stable enzyme expressed in all of the cell types. The LDH is rapidly released into the supernatant cell culture when the plasma membrane was damaged. Following the instructions of the manufacturer, the Cytotoxicity LDH Assay Kit-WST (Donjindo) was used to detect LDH release, while the supernatant from the NSCs was collected after treatment with 100 μmol/L VPA for 48 hours. After normalizing to the medium-only control, the data were expressed as the mean±SEM from the three independent experiments (3 wells per condition).

To obtain the single NSCs, the neurospheres were dissociated chemically using Accutase (Gibco). Using the appropriate concentration of VPA, the cells were incubated for 48 hours and dispensed into laminin and poly-L-ornithine coated 24-well chamber slides in triplicate for every treatment. The NSCs were fixed and treated by PCNA antibody (Proteintech; dilution 1:400) overnight at 4 °C.

The NSCs were treated with 20 μmol/L BrdU (Sigma, USA) for 2 hours, fixed and acid-treated, followed by rat anti-BrdU antibody (Abcam, USA; dilution 1:400) incubation overnight at 4 °C. Quantitative studies were based on three replicates.

Primary neurosphere and secondary neurosphere assay

The NSCs were cultured in a 60-mm dish at a density of 2×10^5 cells/mL, and 100 μmol/L VPA was applied for 48 hours. Then, the diameter of the neurospheres was measured, and the number of neurospheres was counted to evaluate the proliferation activity of cells on day 2. The neurospheres in 3 random fields of view throughout the entire flask were recorded and photographed using an inverted light microscope (Nikon).

To generate secondary neurospheres, on the 7th day after initiating the primary culture, the primary neurospheres including control and VPA treatment group were dissociated with Accutase at 37 °C for 3 minutes. After centrifugation, cells were diluted to 2×10^5 cells/mL using neurosphere media and plated onto 35 mm plates. The secondary neurospheres were counted with VPA for 48 hours. The numbers of neurospheres and their diameters were determined by Image-Pro Plus 6.0. (Media Cybernetics, USA). The total number of neurospheres and their diameters (as a percentage change relative to the control values) were compared among groups.

NSCs cocultured with VPA-treated OGD-injured cell groups

On the 10th day, the primary cultured hippocampus neurons were exposed to hypoxia in glucose-free PBS buffer. To establish the OGD injury model, the

cultured neurons were placed in an oxygen deprivation box and incubated with 95% N_2 and 5% CO_2 at 37 °C for half an hour. The non-OGD control neurons were kept under normal culture conditions for half an hour after being transferred to PBS with glucose. For the glucose and oxygen treatments, the cells were transferred for 48 hours with 5% CO_2 at 37 °C to the Neurobasal/B-27 cultured medium subsequent to OGD treatment.

The coculture system was set up in the 24-well plates hanging insert 0.4 μm PET (Millipore). In the lower chamber of the trans-well plates, the primary cultured hippocampus neurons were plated. In the upper insert chambers, the NSCs, VPA, and the NSCs cocultured with 100 μmol/L VPA were placed after the OGD or the control treatment. The cells were cocultured for 48 hours and then used in subsequent experiments.

Semi-quantitative reverse transcriptase PCR and real-time quantitative PCR

According to the instructions of the manufacturer, the total RNA was extracted from expanded neurospheres with Trizol (Life Technologies, USA) and later reverse transcripted with HiScript Q Select RT SuperMix (Vazyme, China). Following the instructions of the manufacturer, by using the Taq Polymerase (Vazyme), the semi-quantitative reverse transcriptase PCR (RT-PCR) analyses were conducted. The program was as follows: 35 cycles of denaturation for 30 seconds at 95 °C, annealing for 30 seconds at 55 °C, and elongation for 1 minute at 72 °C. Using the 1% agarose gel electrophoresis, the amplified PCR products were analyzed. On a 1% gel at 100 V, the products were electrophoresed. Primers used in this study were summed in ***Table 1***.

Using a LightCycler 96 system (Roche, Switzerland), the measurement of the *Tgfb1* mRNA levels was done using the real-time quantitative PCR. By measuring the binding of the fluorescence dye SYBR Green (Vazyme) to the double-stranded DNA, the amplification was monitored and analyzed. The primers used in this study were summed in ***Table 1***. *Gapdh* was used as an internal control to normalize each primer group.

RNA-Seq and differential gene expression analysis

Total RNA was extracted from normal and paired VPA treated (100 μmol/L for 48 hours) NSCs. RNA sequencing was performed at Nanjing Vazyme BioTech Co., Ltd. Transcript abundance was quantified as fragments per kilo base of exon per million fragments mapped. Differential expression analysis was performed using paired *t*-tests, and genes with a nominal *P*-value <0.05 were determined to be differentially expressed. Enriched Gene Ontology (GO) terms were identified with ToppGene (https://toppgene.cchmc.org/).

Statistical analysis

The results are presented as the mean±SEM. All the data were statistically analysed using GraphPad Prism software. The normally distributed data were analyzed using the parametric unpaired two tailed *t*-test for between-group comparisons, and the nonparametric unpaired two-tailed Mann-Whitney test for between-group comparisons were used to analyze the data with non-normal distribution. For multiple comparisons amongst more than two groups, one-way ANOVA or two-way ANOVA followed by Bonferroni's test were used to analyze the statistical differences.

Results

Bio-characteristics of NSCs and effect of VPA on NSCs

In the conventional NSC medium, the rat NSCs were isolated from the cerebral cortex of E12.5–14.5 embryos and cultured. On the 7th day after initiating the primary culture, the cells cultured in bFGF and

Table 1 **Primers for quantitative reverse transcription PCR analysis**

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Nes	GGTAGGGCTAGAGGACCCAA	TGGGCAATTCAAGGATCCCC
Sox2	AACCGATGCACCGCTACGA	TGCTGCGAGTAGGACATGCTG
Tgfb1	CACCATCCATGACATGAACC	TCATGTTGGACAACTGCTCC
Vit	TGTAAATGCTTCAGAACCTACCAGTC	TCCCATAAACGCTCAATGCTTTCC
Thbs1	GCCAGATGACAAGTTCCAAG	CTGACACCACTTGCTGCTTC
Cola1	TGGTTTCCCTGGTGCTGATG	CTGCCAGTGAGACCCTTGG
Gapdh	ATCAAGAAGGTGGTGAAGCA	AAGGTG GAAGAATGGGAGTTG

EGF supplements had proliferated and formed neurospheres, exhibiting a regular round shape (***Fig. 1A***). The levels of cellular marker proteins and genes of the NSCs were observed to determine the properties of the NSCs under the present culture conditions. Co-immunostaining showed for Nestin and SOX-2 were highly expressed in our cultured NSCs (***Fig. 1A***). Strong expressions of *Sox2* and *Nes* (the gene encoding Nestin) in the NSCs were showed in the semi-quantitative RT-PCR analysis (***Fig. 1B***). The gene expression patterns of the NSCs were found to be indistinguishable from those of the cultured NSCs.

To figure out whether VPA regulated the neurosphere viability of NSCs *in vitro*, the cultured NSCs were subjected to VPA exposure. The concentration effect curve of VPA was firstly explored by CCK8 assay. The concentration effect curve of VPA was firstly explored by CCK8 assay. As shown in ***Fig. 1C*** and ***D*** , the VPA treatment for 48 hours enhanced neurosphere viability in a concentration-dependent manner and 50% effective concentration (EC_{50}) occurred between 10 and 100 μmol/L. Hence, the 100 μmol/L VPA treatment for 48 hours was selected for the subsequent experiments. Based on the LDH release, the cytotoxicity of the VPA was also assessed. The treatment of 100 μmol/L VPA for 48 hours did not increase LDH release compared with the control group (***Fig. 1E***).

Effects of VPA on NSC viability and neurosphere formation

The ERK pathway-dependent cortical neurogenesis and neuronal growth have been reported to be promoted by valproate in a time- and concentration-dependent manner[10]. After 100 μmol/L VPA treatment for 24, 48, and 72 hours, the images of neurospheres formation were captured (***Fig. 2A***). The results of CCK8 assay demonstrated that the viability of NSCs was increased in a time-dependent manner in the control group, while it was further enhanced by the 100 μmol/L VPA treatment for 48 hours or 72 hours (***Fig. 2B***). Moreover, the treatment with different concentrations of VPA enhanced the viability of NSCs in the same manner except that the cell viability was significantly suppressed by VPA at the concentration of 10 000 μmol/L (***Fig. 2B***).

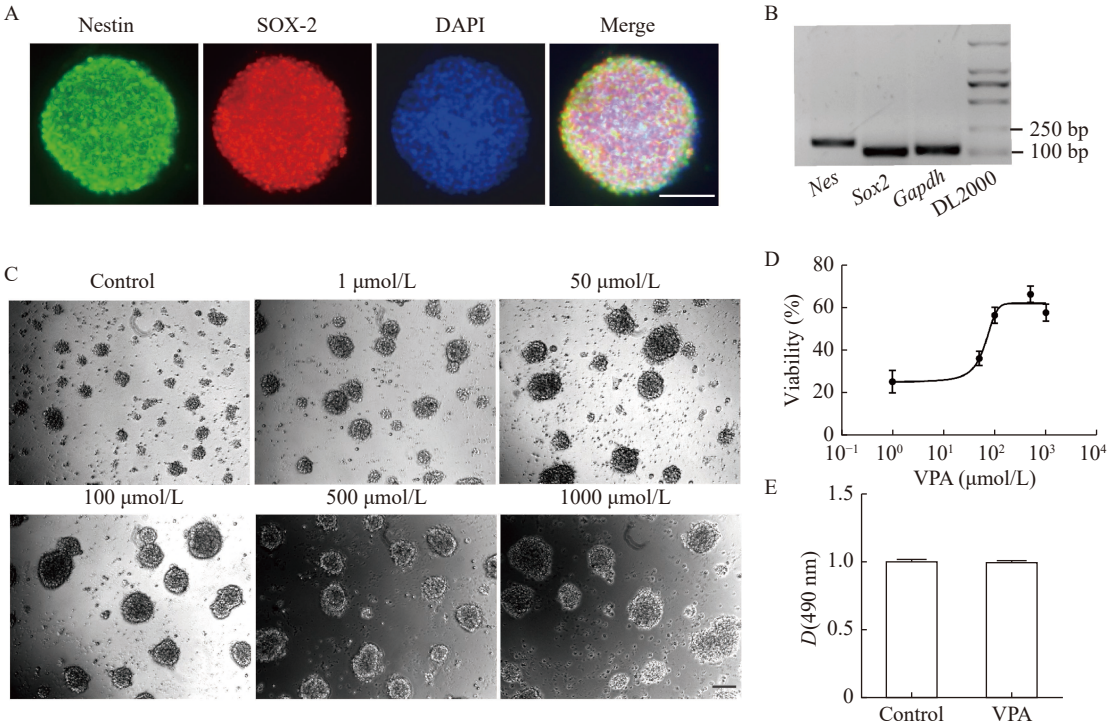

Fig. 1 **Identification of cultured NSCs and the effective concentration of VPA.** Identification of NSCs by immunofluorescence and semiquantitative RT-PCR. A: Neurospheres were immunostained with Nestin (green) and SOX-2 (red), and nuclei were counterstained with DAPI (blue). Scale bar: 50 μm. B: Representative PCR products of *Nes* and *Sox2*; *Gapdh* was used as a loading control. C: Representative images of NSCs with the indicated concentrations of VPA (0, 1, 50, 100, 500, and 1000 μmol/L) treatment for 48 hours. Scale bar: 50 μm. D: The EC_{50} values indicating cell viability in NSCs. EC_{50} values were calculated using a mathematic function antilog of values provided by sigmoidal dose-response curves (N=3). E: Cytotoxicity assay of NSCs based on LDH release after the NSCs were treated with 100 μmol/L VPA for 48 hours (N=3, n=10). Data are presented as the mean±SEM; statistical analyses were performed using unpaired two-tailed t-test. EC_{50}: 50% effective concentration; LDH: lactate dehydrogenase; N: experimental repetition times; n: total number of experimental samples.

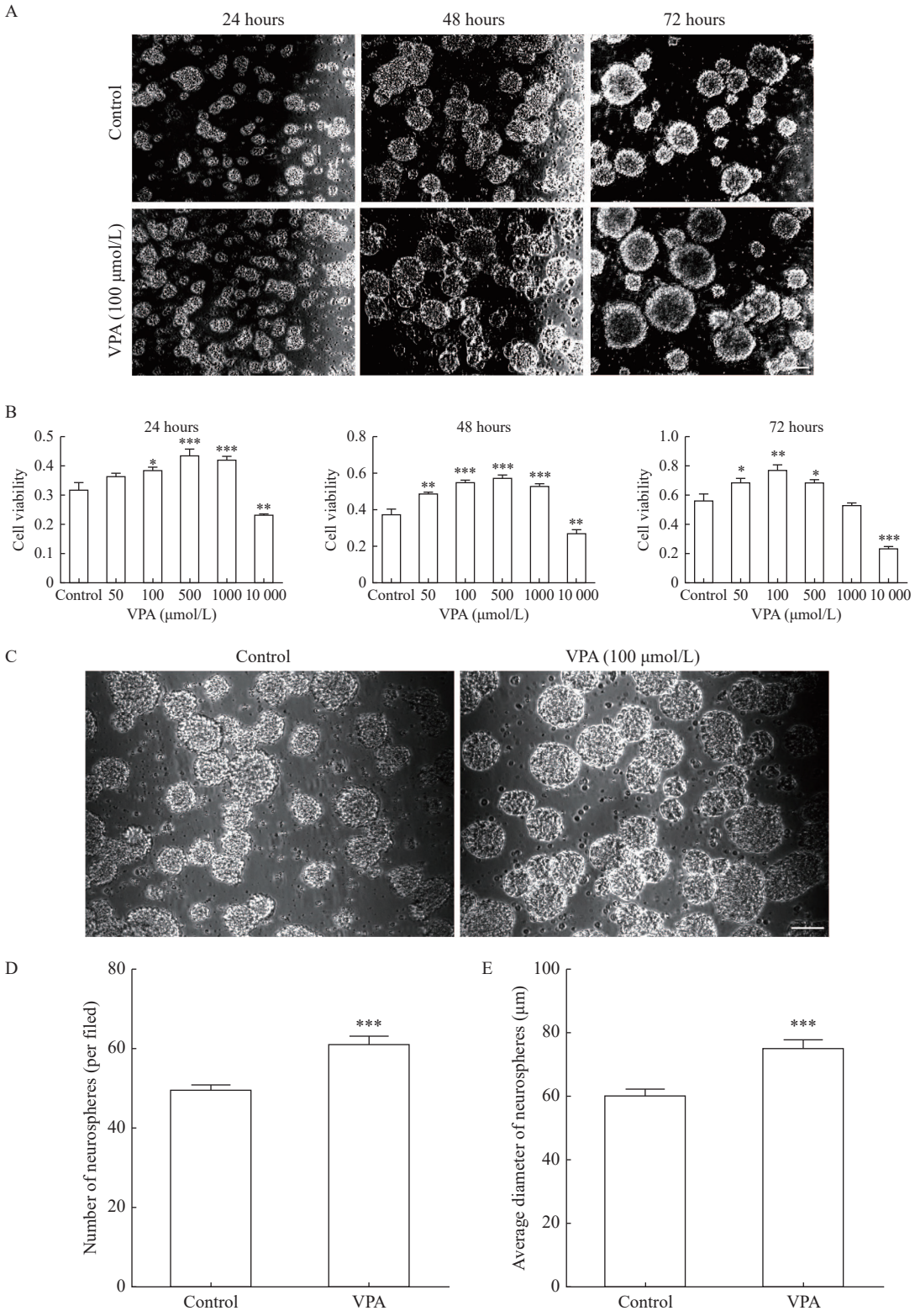

Fig. 2 **VPA treatment enhanced NSCs viability and neurosphere formation in culture.** A: Representative images of neurospheres treated with 100 μmol/L VPA for 24, 48, and 72 hours. Scale bar: 50 μm. B: The effects of VPA on NSCs viability were evaluated using CCK8 assays (N=4, n=14). NSCs were treated with VPA (0, 50, 100, 500, 1000, and 10 000 μmol/L). Growth trend of NSCs was shown for each treatment at 24, 48, and 72 hours (N=4, n=14). Data are presented as the mean±SEM; statistical analyses were performed using one-way ANOVA; $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$ compared with untreated group. C: Representative images of NSCs with 100 μmol/L VPA treatment for 48 hours by the inverted fluorescence microscope. Scale bar: 50 μm. D: Quantitation of the number of neurospheres per field. The NSCs were treated with 100 μmol/L VPA for 48 hours (N=3, n=53). Data are presented as the mean±SEM; statistical analyses were performed using unpaired two-tailed t-test; $^{***}P<0.001$ compared with untreated group. E: The average diameter of neurospheres. The NSCs were treated with 100 μmol/L VPA for 48 hours (N=3, n=26–37). Data are presented as the mean±SEM; statistical analyses were performed using unpaired two-tailed Mann-Whitney test; $^{***}P<0.001$ compared with untreated group. N: experimental repetition times; n: total number of experimental samples.

We performed the neurosphere forming assays to identify the effects of VPA in NSCs. Neurospheres were cultured with or without 100 μmol/L VPA for 48 hours. More neurospheres per field were observed in the VPA group compared with the control group (***Fig. 2C*** and ***D***, 60.9±2.2 *vs.* 49.6±1.2). Moreover, the diameter of the neuropheres in the VPA group was significantly larger than that in the control group (***Fig. 2C*** and ***E***, [75.2±2.6] μm *vs.* [60.3±1.9] μm). These results indicated that VPA treatment increased the number and size of the formed neurospheres.

Role of VPA in NSC proliferation

Proliferating cell nuclear antigen (PCNA), a molecular marker, was used for proliferation assay. Immunofluorescent staining showed that compared with the control group, the VPA group had significantly increased number of $PCNA^+$ cells (***Fig. 3A*** and ***B***, [37.4±2.5]% *vs.* [30.0±2.6]%).

Then after the 20 μmol/L BrdU treatment for 2 hours, the NSCs were double-stained against Nestin and BrdU. We observed that VPA administration in NSCs noticeably improved the number of $BrdU^+$ cells compared with the control group (***Fig. 3C*** and ***D***, [77.7±5.1]% *vs.* [55.4±7.4]%). Collectively, these data indicated that VPA treatment promoted NSC proliferation.

VPA improved the secondary neurosphere formation

To study the effects of VPA treatment on the self-renewal ability of NSCs, a secondary neurosphere assay was developed. The neurospheres were digested by Accutase and cultured again in the complete medium, with a subset forming secondary neurospheres. Exogenous addition of VPA increased the size of secondary neurospheres (***Fig. 4A*** and ***B***, [56.4±1.8] μm *vs.* [43.2±1.6] μm).

VPA treatment of NSCs reduced the apoptosis of OGD-injured hippocampus neurons

To explore potential the beneficial effects of VPA-treated NSCs on the OGD-injured hippocampus neurons, the coculture system was set up. To assess the beneficial effect of VPA treatment on NSCs to the injured hippocampus neurons and the OGD-injured model, the apoptotic cells were stained by propidium iodide (PI) (***Fig. 5A***). As shown in ***Fig. 5B***, the PI-

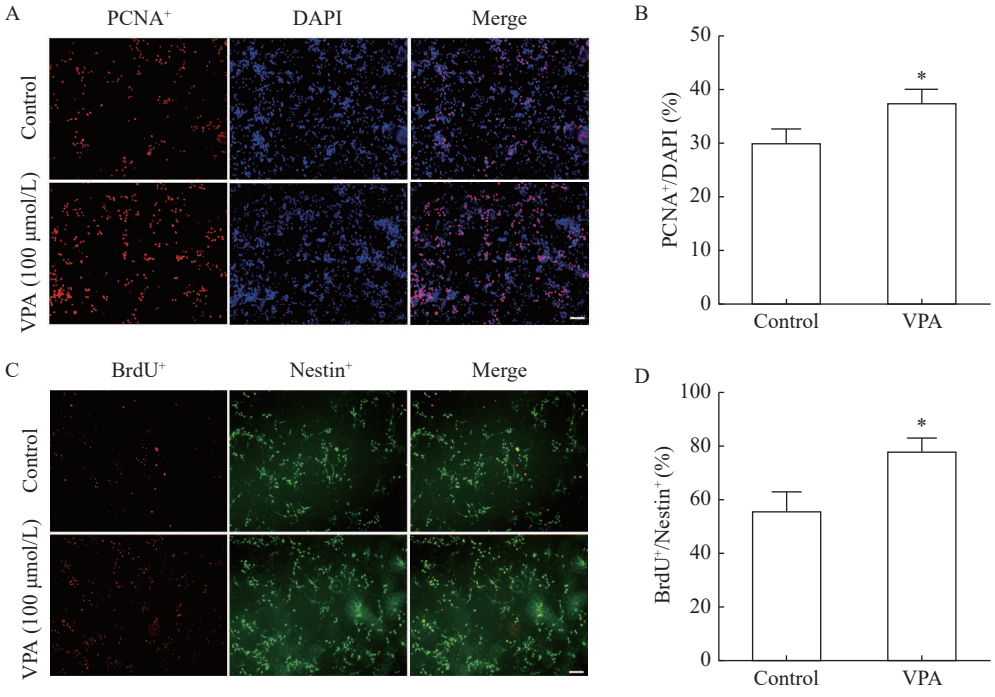

Fig. 3 **VPA promoted NSC proliferation.** A: PCNA (red) and DAPI (blue) immunofluorescent staining of cultured adherent NSCs after treatment with 100 μmol/L VPA for 48 hours. Scale bar: 50 μm. B: Quantification of the number of proliferating NSCs ($PCNA^+$). The adherent NSCs were treated with 100 μmol/L VPA for 48 hours (N=3, n=17–18). C: BrdU (red) and Nestin (green) immunofluorescent staining of cultured adherent NSCs after treatment with 100 μmol/L VPA for 48 hours. Scale bar: 50 μm. D: Quantification of the number of proliferating NSCs ($BrdU^+$). The adherent NSCs were treated with 100 μmol/L VPA for 48 hours (N=3, n=12–15). Data are presented as the mean±SEM; statistical analyses were performed using unpaired two-tailed t-test; $^*P<0.05$ compared with untreated group. PCNA: proliferating cell nuclear antigen; BrdU: 5-bromo-2'-deoxyuridine. N: experimental repetition times; n: total number of experimental samples.

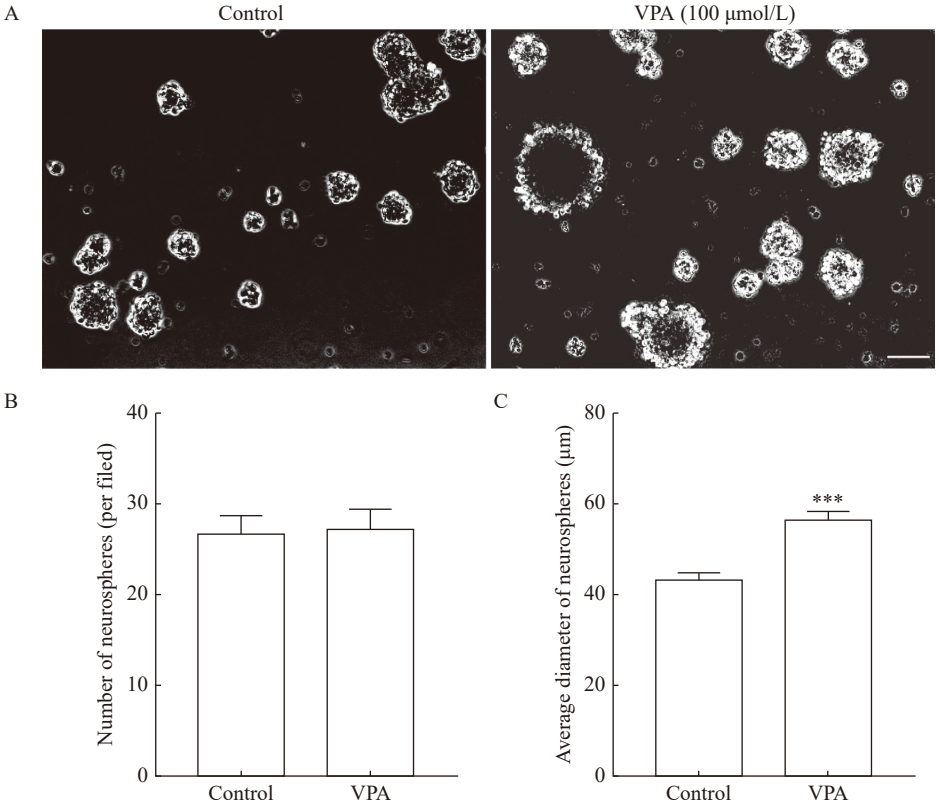

Fig. 4 **Secondary neurospheres formation *in vitro*.** The secondary neurospheres were treated with 100 μmol/L VPA for 48 hours. A: Representative images of secondary neurospheres. Scale bar: 50 μm. B: Quantitation of the number of secondary neurospheres per field (N=3, n=90–102). C: Quantitation of the average diameter of neurospheres (N=3, n=59–72). Data are presented as the mean±SEM; statistical analyses were performed using unpaired two-tailed t-test; $^{***}P<0.001$ compared with untreated group. N: experimental repetition times; n: total number of experimental samples.

positive cells were increased to (58.5±6.1)% after OGD treatment, and the application of VPA alone exerted no effects on OGD-induced cell death ([51.3±6.4]%). However, the coculture of NSCs with the hippocampus neurons showed protective effects on OGD-induced cell death ([46.1±1.5]%, $P<0.01$ compared with OGD group). Moreover, the protective effects of NSCs was further improved by the application of VPA ([30.3±3.2]%, $P<0.01$ compared with OGD+NSCs group). Together, the cocultured data showed that VPA-treated NSCs could further attenuate the damage of neurons induced by OGD.

Transcriptomic changes of NSCs in response to VPA and verification of candidate genes

To systematically investigate the effect of VPA on NSCs, we performed RNA-seq of matched control and VPA-treated NSCs. We identified 454 significantly differentially expressed genes: 180 were increased in VPA-treated groups, and 274 were decreased. The differentially expressed genes that were upregulated by VPA (*e.g.*, *Tgfb1* and *Vit*) were enriched in GO terms such as cell proliferation and growth, as illustrated in the expression heatmap (***Fig. 6A***), suggesting that VPA treatment is responsible for facilitating the proliferation and the development of NSCs. Among the identified candidate genes, *Tgfb1* particularly aroused our interest, as it is expressed in the brain and has been demonstrated to directly influence the production of neural crest cell fates[13]. To evaluate the RNA-Seq results and investigate the expression of *Tgfb1* in NSCs with or without VPA treatment, *Tgfb1* and the reference genes showing identical expression were selected for RT-PCR analysis. Consistent with the microarray data, our RT-PCR analyses confirmed an increased expression of *Tgfb1* mRNA, and the mRNA levels of *Vit* were also dramatically increased in VPA-treated NSCs compared with the control, while the irrelevant mRNA levels of *Thbs1* and *Cola1* were not changed before and after VPA treatment (***Fig. 6B*** and ***C***).

TGFβ1 regulated positive effects of VPA on neurophere formation and proliferation of NSC

To verify the contribution of TGFβ1 to the modulation of VPA treatment on the NSCs proliferation, the TGFβ1 receptor kinase activity inhibitor of SB431542 was added to NSCs, then each group was detected by a CCK8 assay. As shown in

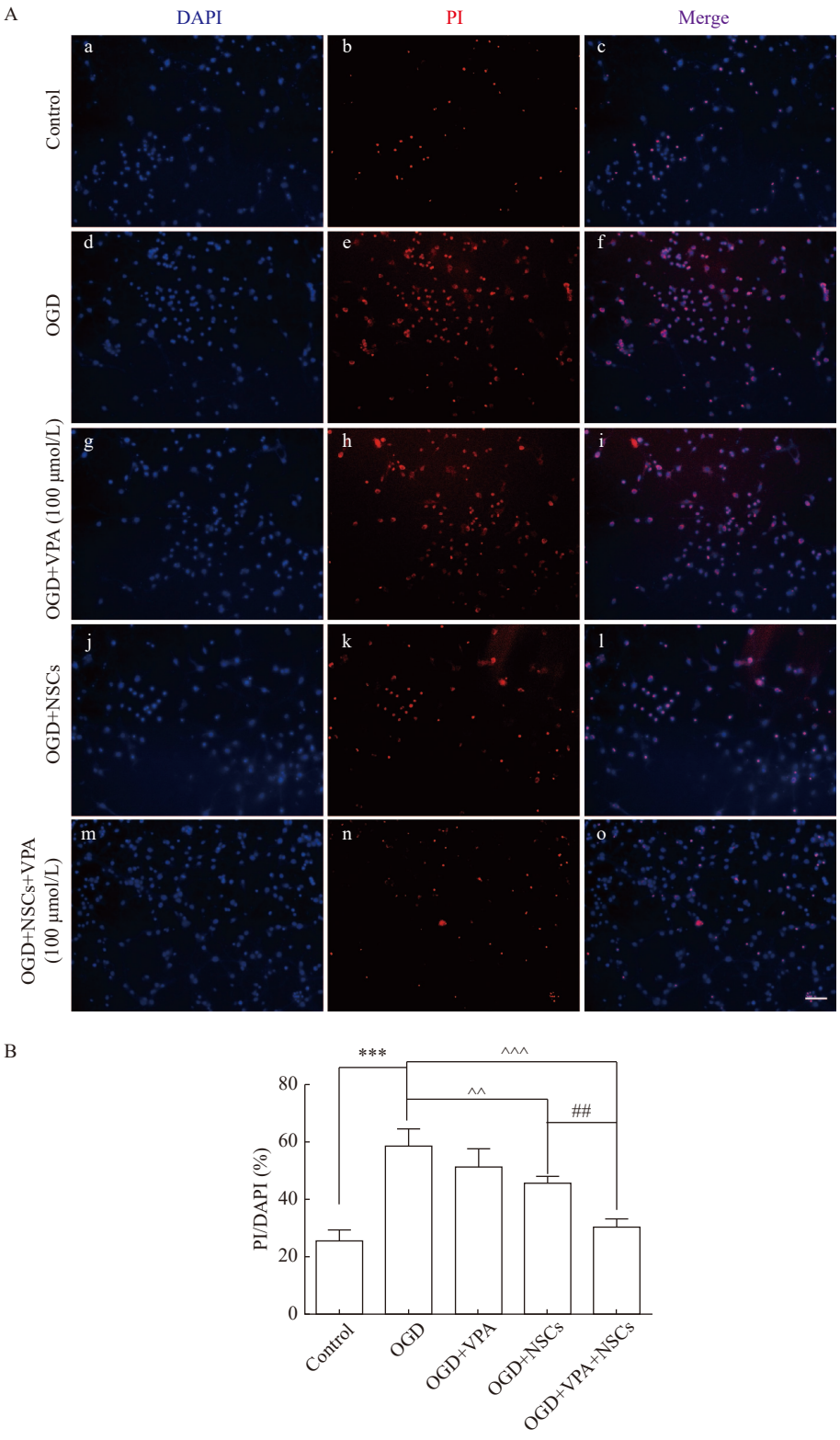

Fig. 5 **Repairing effects of the cocultured NSCs treated with VPA on OGD-injured neurons.** A: DAPI/PI double staining of neurons. The experimental groups included the control, OGD-injured neurons, 100 μmol/L VPA-treated OGD-injured neurons for 48 hours, NSCs-treated OGD-injured neurons for 48 hours, and NSCs coupled with 100 μmol/L VPA-treated OGD-injured neurons for 48 hours. a, d, g, j, and m: DAPI-positive cells in neurons. b, e, h, k, and n: PI-positive cells in neurons. c, f, i, l, and o: DAPI-positive cells and PI-positive cells merged channels. Scale bar: 50 μm. B: Quantitative analyses of PI-positive cells in control, OGD-injured neurons, 100 μmol/L VPA-treated OGD-injured neurons for 48 hours, NSCs-treated OGD-injured neurons for 48 hours, and NSCs coupled with 100 μmol/L VPA-treated OGD-injured neuros for 48 hours. PI-positive cells/DAPI are shown (N=3, n=9). Data are presented as the mean±SEM; statistical analyses were performed using one-way ANOVA; $^{***}P<0.001$ compared with the control group; $^{\wedge\wedge}P<0.01$ compared with the OGD-injured group; $^{\wedge\wedge\wedge}P<0.001$ compared with the OGD-injured group; $^{\#\#}P<0.01$ compared with the NSCs-treated OGD-injured group. N: experimental repetition times; n: total number of experimental samples.

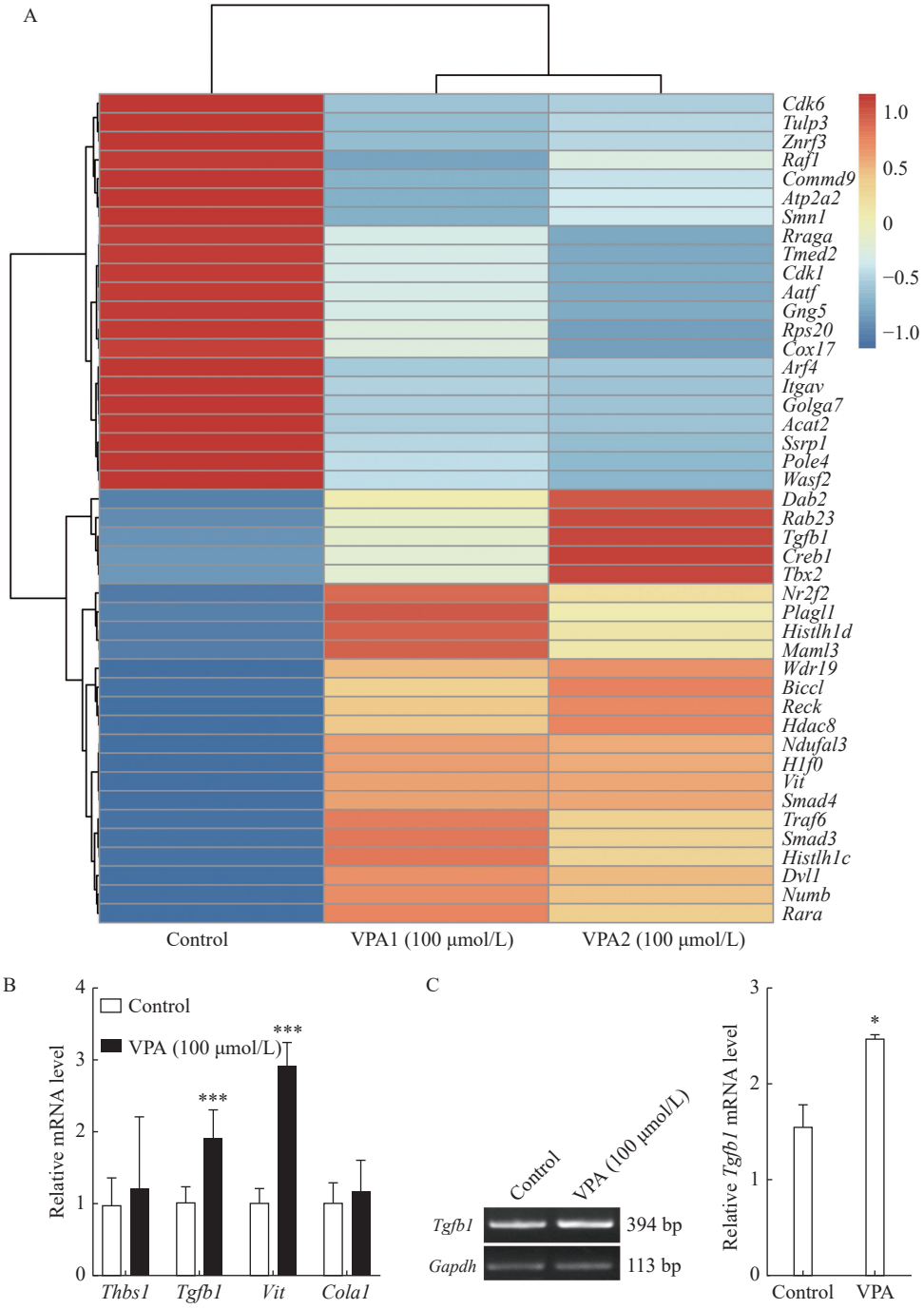

Fig. 6 **Transcriptomic profiles in NSCs following VPA treatment.** A: Hierarchical clustering heatmap showing the relative $\log_2$(fold change) of 44 differentially expressed genes enriched in GO terms cell proliferation and growth. Blue and red suggests $\log_2$(fold change)<0 and $\log_2$(fold change)>0 in group of NSCs treated with 100 μmol/L VPA for 48 hours (*vs.* control), respectively (n=6). B: The NSCs incubated with 100 μmol/L VPA for 48 hours. The *Thbs1*, *Tgfb1*, *Vit*, and *Cola1* mRNA levels were analyzed by quantitative RT-PCR. Data are presented as the mean±SEM; statistical analyses were performed using unpaired two-tailed *t*-test; $^{***}P$<0.001 compared with untreated group. C: Semi quantitative RT-PCR analysis of TGFβ1 from NSCs treated with 100 μmol/L VPA for 48 hours. Data are presented as the mean±SEM; statistical analyses were performed using unpaired two-tailed *t*-test; $^{*}P$<0.05 compared with the control group. *n*: total number of experimental samples.

Fig. 7A, the enhanced viability of NSCs by VPA was blocked by the treatment of 1 μmol/L or 5 μmol/L of SB431542. And the diameter of the neuropheres in the VPA+SB431542 group was remarkably smaller than that in the VPA group (***Fig. 7B*** and ***C***, [28.3±0.7] μm *vs.* [35.3±1.2] μm), but it had no difference with the control group ([28.3±0.7] μm *vs.* control group: [27.7±0.7] μm).

Furthermore, we observed the effect of SB431542 on VPA-treated NSCs by BrdU application for 2

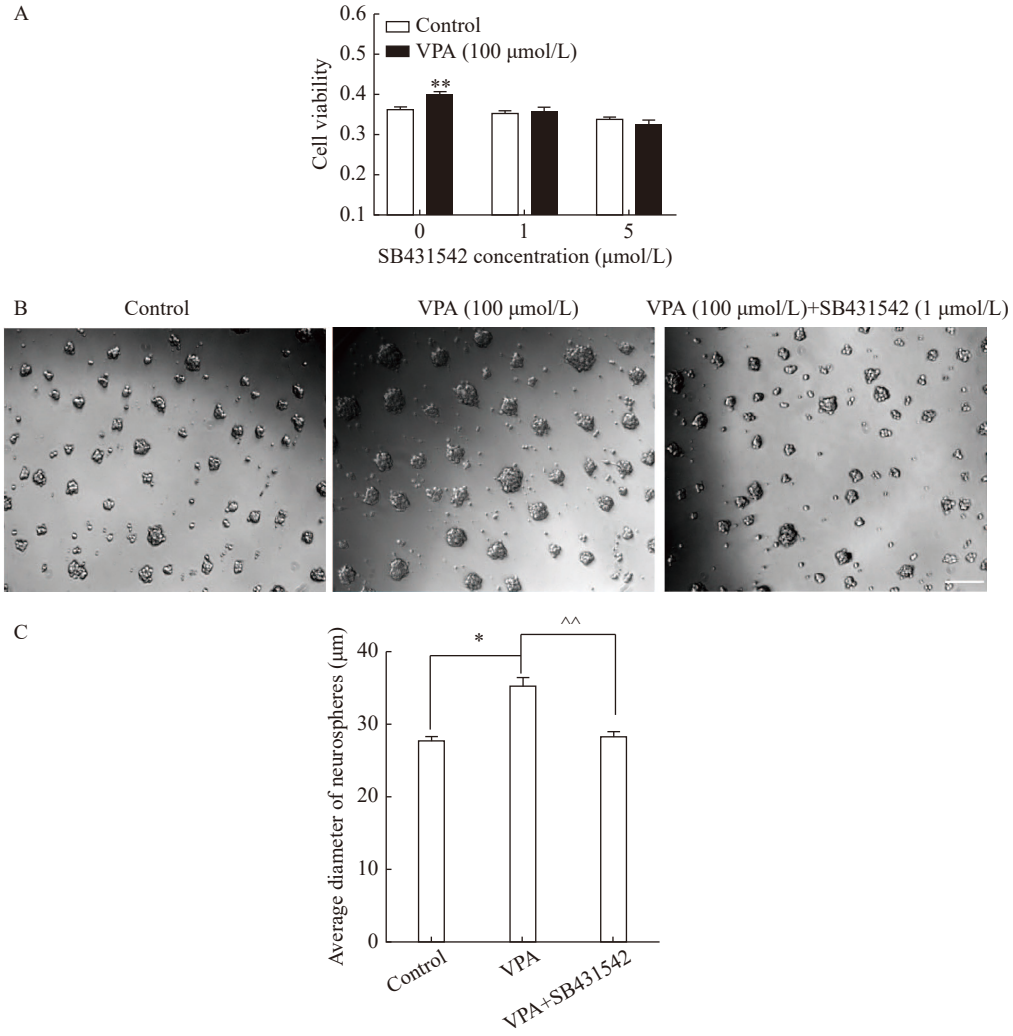

Fig. 7 **SB431542 reduced the VPA mediated increase of the size of secondary neurospheres.** A: The indicated concentration of SB431542 (1 μmol/L or 5 μmol/L) was added to the medium, and a CCK8 assay was conducted after 48 hours. Data are presented as the mean±SEM (*N*=3); statistical analyses were performed using two-way ANOVA; $^{**}P<0.01$ compared with untreated group. B: Representative images of neurospheres from the control group, VPA (100 μmol/L) treatment group or VPA (100 μmol/L) + SB431542 (1 μmol/L) group. Scale bar: 50 μm. C: Quantitation of the average diameter of secondary neurospheres per field after treatment with VPA (100 μmol/L) or VPA (100 μmol/L) + SB431542 (1 μmol/L) for 48 hours (*N*=3, *n*=37–41). Data are presented as the means±SEM; statistical analyses were performed using one-way ANOVA; $^{*}P<0.05$ compared with the control group; $^{\wedge\wedge}P<0.01$ compared with the VPA group. *N*: experimental repetition times; *n*: total number of experimental samples.

hours. Then, the NSCs were double-stained against Nestin and BrdU. The administration of SB431542 with VPA in NSCs markedly reduced the number of $BrdU^+$ cells compared with the group using VPA alone (***Fig. 8A*** and ***B***, [72.4±5.4]% *vs.* VPA group: [92.8±2.1]%), but had no difference with the control group ([72.4±5.4]% *vs.* [77.1±3.0]%). Collectively, these data indicated that the positive effects of VPA on neuropheres formation and proliferation of NSCs might be attributed to the activation of TGFβ1 signaling.

Discussion

VPA concentration and exposure period were significantly corrected with the cell survival rate. A previous study reported that VPA (1 mmol/L) simultaneously induced differentiation and decreased proliferation of embryonic day 14 (E14) rat cerebral cortex NPCs under adherent conditions in culture[9]. VPA (0.2 to 0.5 mmol/L) increases the proliferation of NPCs under adherent conditions in culture from rat E14 brain[14]. Additionally, it has been reported that valproate promotes E18 cortical neuronal growth and neurogenesis in a time- and concentration-dependent manner[10]. A proliferative effect of VPA (1 to 2 mmol/L) on the NSCs in the embryonic hindbrain was observed in the earlier studies. Contrarily, in the adult zebrafish optic tectum, VPA (500 μmol/L) reduced cell proliferation[11]. When separated from its niche *in*

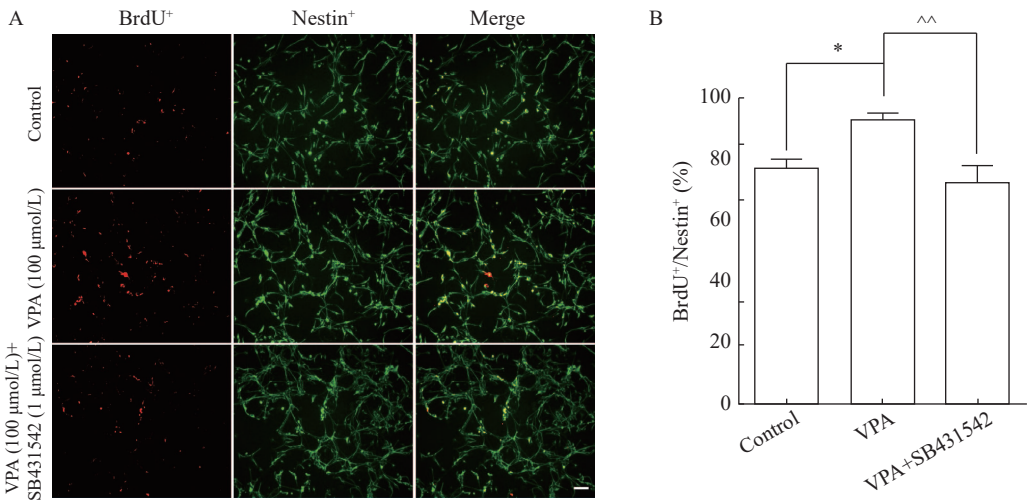

Fig. 8 **SB431542 inhibited the VPA mediated proliferation of NSCs.** Cultured adherent NSCs were treated with VPA (100 μmol/L) or VPA (100 μmol/L) + SB431542 (1 μmol/L) for 48 hours. A: Representative images from BrdU (red) and Nestin (green) immunofluorescent staining. Scale bar: 50 μm. B: Quantification of the number of proliferating NSCs (BrdU+) (*N*=3, *n*=8). Data are presented as the mean±SEM; statistical analyses were performed using one-way ANOVA; **P*<0.05 compared with the control group; ^^*P*<0.01 compared with the VPA group. *N*: experimental repetition times; *n*: total number of experimental samples.

vivo, the sphere-forming assays evaluate the potential of a cell as a stem cell[2]. The neurosphere formation by adult subventricular cells was inhibition of the VPA (1 mmol/L)[12]. Our research revealed that VPA increased neurosphere formation and NSC proliferation in a time- and dose-dependent. While the cell viability was significantly suppressed by VPA at the concentration of 10 mmol/L, the CCK8 assay also revealed that 100 μmol/L VPA increased neurosphere formation. The dose-responses make proper connection with the emerging principles of hormesis. Hormesis is a biphasic dose-response relationship characterized by low dose stimulation and high dose inhibition[15]. Hormesis can be used as a new approach in the investigation and translational therapy of amyotrophic lateral sclerosis in experimental and clinical research, thus showing a neuroprotective effect[16].

For replacing lost neurons and rescuing injured neurons subsequent to the ischemic cascade, the stem cell transplantation is considered as a potential treatment based on the current research on stem cells[17]. Moreover, cotreatment strategies were developed for neural regeneration by using NSCs coupled with selective drug therapy. According to our results, VPA promoted the proliferation of NSCs and might be beneficial for controlling the damage caused by OGD treatment. Increased differentiation of the transplanted NSCs was found to be induced by the stimulation of the increased growth factor production by the OGD lesion, as indicated by several studies. Both glia and neurons differentiated from the NSCs seem to have the potential to replace injured nerve cells[18]. Nonetheless, to identify the role of VPA in the differentiation of NSCs, further studies are needed.

Preconditioning ischemia is a typical representative of hormesis in which cells are exposed to moderate transient stress to protect themselves from more severe stress. Vitagenes are defined as cell survival stress responsive genes which give play to free radicals' detoxification and rescue the mitochondrial function under oxidative stress in the brain. Sirtuin-1 (Class Ⅲ HDACs), also known as a vitagene, helps to maintain protein homeostasis and cell redox under some pathological conditions[19]. The hormesis and vitagenes can be used to address various neurodegenerative diseases. Sirtuin-1 and related hormetic signaling pathways have been reported to cooperate with transcriptional regulators to promote cell survival[20]. The hormetic-preconditioning patterns and outcomes were supported to address the aging-associated neurodegenerative diseases including the Alzheimer's disease, Parkinson's disease, and Huntington's disease[21]. Based on the above results, preconditioning of VPA on OGD-injured model is valuable for our further research.

RNA-Seq analysis was performed to examine comprehensive changes in gene expression following VPA stimulation in NSCs. Our results revealed that the VPA-dependent genes were highly enriched for cell proliferation and growth, VIT has relatively high homology to Akhirin (AKH). AKH was expressed specifically in the ependymal cells of the central canal spinal cord during development. AKH regulated the

differentiation and proliferation of NSCs/NPCs. In mice lacking the AKH gene, the proliferation of cells was decreased and cell differentiation was altered in the central canal area. The size of neurospheres was reduced in the *AKH*$^{-/-}$ mice spinal cord[22]. As it was expressed in the brain and demonstrated to directly influence the production of a particular neural crest cell fates, the *TGFB1* was of our particular interest, amongst all the identified candidate genes[13]. Having a crucial role in proliferation, morphology and cell communication, the TGFβ1 was confirmed to activate Smad3/Smad4 to promote the gap junction formation in the chondrocytes[23]. As demonstrated by the reduced infarct size, the experimental results indicated that TGFβ1 and Smad3 played a protective role against ischemic strokes[24]. TGFβ was found to increase the cell size and the protein content during EMT[25]. Previous studies have shown that butyrate (an HDACi) activates TGFβ1 expression in intestinal epithelial cell lines[26]. Recently, TSA (another HDACi) was shown to upregulate Scx expression through inhibiting HDAC1 and HDAC3, increasing the H3K27Ac level of TGFβ1 and the TGFβ2 genome region in ScxGFP mouse-derived TSPCs[27]. Consistent with the microarray data, our RT-PCR analyses confirmed the significantly enhanced expression of *Tgfb1* mRNA. SB431542, a selective and potent inhibitor of the TGFβ pathway, can remove TGFβ signal from the culture medium and inhibit the activation of SMAD, which results in the differentiation of hESCs along the neuroectoderm lineage. Blocking TGFβ signaling caused differentiation, indicating that TGFβ signaling was required to maintain the hESC state[28]. SB431542 was added to NSCs, the co-application of SB431542 with VPA blocked the enhanced effects of VPA on the neurosphere formation and proliferation of NSCs, indicating that TGFβ signaling pathway, at least, partly mediates the role of VPA on NSCs.

In summary, our study showed that VPA treatment could promote neurosphere formation and NSC proliferation, and the activation of TGFβ1 signaling might be involved in this process. Moreover, NSCs treated with VPA partly reduced the apoptosis of OGD-injured neurons *in vitro*. Overall, our study has laid a basis for further exploration of VPA in neuroscience and provides a new therapeutic approach for nerve injury.

Acknowledgments

This work was supported by grants from the Key R&D Program of Jiangsu Province (Grant No. 2017CX010) and the National Natural Science Foundation of China (Grant No. 81973308) to J.G., and the Nanjing Science and Technology Development Program (Grant No. 201402021) to H.L.

References

[1] Lindvall O, Kokaia Z. Stem cells for the treatment of neurological disorders[J]. *Nature,* 2006, 441(7097): 1094–1096.

[2] Pastrana E, Silva-Vargas V, Doetsch F. Eyes wide open: a critical review of sphere-formation as an assay for stem cells[J]. *Cell Stem Cell,* 2011, 8(5): 486–498.

[3] Tang H, Zhu J. Epigenetics and neural stem cell commitment[J]. *Neurosci Bull,* 2007, 23(4): 241–248.

[4] Sun G, Fu C, Shen C, et al. Histone deacetylases in neural stem cells and induced pluripotent stem cells[J]. *J Biomed Biotechnol,* 2011, 2011: 835968.

[5] Lagger G, O'Carroll D, Rembold M, et al. Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression[J]. *EMBO J,* 2002, 21(11): 2672–2681.

[6] Montgomery RL, Hsieh J, Barbosa AC, et al. Histone deacetylases 1 and 2 control the progression of neural precursors to neurons during brain development[J]. *Proc Natl Acad Sci USA,* 2009, 106(19): 7876–7881.

[7] Sun G, Yu RT, Evans RM, et al. Orphan nuclear receptor TLX recruits histone deacetylases to repress transcription and regulate neural stem cell proliferation[J]. *Proc Natl Acad Sci USA,* 2007, 104(39): 15282–15287.

[8] Qiu X, Xiao X, Li N, et al. Histone deacetylases inhibitors (HDACis) as novel therapeutic application in various clinical diseases[J]. *Prog Neuropsychopharmacol Biol Psychiatry,* 2017, 72: 60–72.

[9] Jung GA, Yoon JY, Moon BS, et al. Valproic acid induces differentiation and inhibition of proliferation in neural progenitor cells *via* the beta-catenin-Ras-ERK-p21$^{Cip/WAF1}$ pathway[J]. *BMC Cell Biol,* 2008, 9: 66.

[10] Hao Y, Creson T, Zhang L, et al. Mood stabilizer valproate promotes ERK pathway-dependent cortical neuronal growth and neurogenesis[J]. *J Neurosci,* 2004, 24(29): 6590–6599.

[11] Dozawa M, Kono H, Sato Y, et al. Valproic acid, a histone deacetylase inhibitor, regulates cell proliferation in the adult zebrafish optic tectum[J]. *Dev Dyn,* 2014, 243(11): 1401–1415.

[12] Zhou Q, Dalgard CL, Wynder C, et al. Valproic acid inhibits neurosphere formation by adult subventricular cells by a lithium-sensitive mechanism[J]. *Neurosci Lett,* 2011, 500(3): 202–206.

[13] Dorsky RI, Moon RT, Raible DW. Environmental signals and cell fate specification in premigratory neural crest[J]. *Bioessays,* 2000, 22(8): 708–716.

[14] Go HS, Kim KC, Choi CS, et al. Prenatal exposure to valproic acid increases the neural progenitor cell pool and induces macrocephaly in rat brain *via* a mechanism involving

the GSK-3β/β-catenin pathway[J]. *Neuropharmacology,* 2012, 63(6): 1028–1041.

[15] Calabrese V, Cornelius C, Dinkova-Kostova AT, et al. Cellular stress responses, the hormesis paradigm, and vitagenes: novel targets for therapeutic intervention in neurodegenerative disorders[J]. *Antiox Redox Signal,* 2010, 13(11): 1763–1811.

[16] Calabrese EJ, Calabrese V, Giordano J. Demonstrated hormetic mechanisms putatively subserve riluzole-induced effects in neuroprotection against amyotrophic lateral sclerosis (ALS): implications for research and clinical practice[J]. *Ageing Res Rev,* 2021, 67: 101273.

[17] Martino G, Pluchino S. The therapeutic potential of neural stem cells[J]. *Nat Rev Neurosci,* 2006, 7(5): 395–406.

[18] Liu Q, Fan X, Zhu J, et al. Co-culturing improves the OGD-injured neuron repairing and NSCs differentiation *via* Notch pathway activation[J]. *Neurosci Lett,* 2014, 559: 1–6.

[19] Siracusa R, Scuto M, Fusco R, et al. Anti-inflammatory and anti-oxidant activity of Hidrox® in rotenone-induced parkinson's disease in mice[J]. *Antioxidants (Basel),* 2020, 9(9): 824.

[20] Miquel S, Champ C, Day J, et al. Poor cognitive ageing: Vulnerabilities, mechanisms and the impact of nutritional interventions[J]. *Ageing Res Rev,* 2018, 42: 40–55.

[21] Calabrese E J, Mattson M P, Dhawan G, et al. Hormesis: A potential strategic approach to the treatment of neurodegenerative disease[J]. *Int Rev Neurobiol,* 2020, 155: 271–301.

[22] Abdulhaleem FAM, Song X, Kawano R, et al. Akhirin regulates the proliferation and differentiation of neural stem cells in intact and injured mouse spinal cord[J]. *Dev Neurobiol,* 2015, 75(5): 494–504.

[23] Wang Q, Zhou C, Li X, et al. TGF-β1 promotes gap junctions formation in chondrocytes *via* Smad3/Smad4 signalling[J]. *Cell Prolif,* 2019, 52(2): e12544.

[24] Yu Y, Li J, Zhou H, et al. Functional importance of the TGF-β1/Smad3 signaling pathway in oxygen-glucose-deprived (OGD) microglia and rats with cerebral ischemia[J]. *Int J Biol Macromol,* 2018, 116: 537–544.

[25] Lamouille S, Derynck R. Cell size and invasion in TGF-β-induced epithelial to mesenchymal transition is regulated by activation of the mTOR pathway[J]. *J Cell Biol,* 2007, 178(3): 437–451.

[26] Martin-Gallausiaux C, Béguet-Crespel F, Marinelli L, et al. Butyrate produced by gut commensal bacteria activates *TGF-β1* expression through the transcription factor SP1 in human intestinal epithelial cells[J]. *Sci Rep,* 2018, 8(1): 9742.

[27] Zhang C, Zhang E, Yang L, et al. Histone deacetylase inhibitor treated cell sheet from mouse tendon stem/progenitor cells promotes tendon repair[J]. *Biomaterials,* 2018, 172: 66–82.

[28] James D, Levine AJ, Besser D, et al. TGFβ/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells[J]. *Development,* 2005, 132(6): 1273–1282.