

Original Article

Retinoic acid induced the differentiation of neural stem cells from embryonic spinal cord into functional neurons *in vitro*

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Abstract: Retinoic acid is an important molecular taking part in the development and homeostasis of nervous system. Neural stem cells (NSCs) are pluripotent cells that can differentiate into three main neural cells including neuron, astrocyte and oligodendrocyte. However, whether retinoic acid can induce NSCs derived from embryonic spinal cord differentiating into functional neurons and its efficiency are not clear. In this experiment, NSCs were isolated from embryonic 14 d spinal cord of rats. The growth and neuronal differentiation of NSCs induced by 500 nM RA was examined *in vitro*. It was indicated that compared with the control group, there were more differentiated cells with longer cytodendrites in the medium treated with RA at different time. And more, there were more neuronal marker positive cells in 500 nM RA group than the control group seven days after differentiation. At the same time, the expression of β -tubulin III protein in RA group was higher than those in control group, which was contrary to the expression of astrocyte marker GFAP protein at seven days after differentiation. However the differentiated neurons, whether treated with RA or not both exhibited biological electrical reactivity after stimulated by glutamine. Therefore, these findings indicated that RA could promote growth of cellular dendrites and neuronal differentiation of NSCs, which also induce functional maturation of differentiated neurons finally.

Keywords: Retinoic acid, neural stem cells, differentiation, culture

Introduction

Neural stem cells (NSCs) have the ability to self-renew and differentiate into different types of neural cells, which exhibited a new field for the research of central nervous system. Up to now the mechanism and its signal pathways for regulating NSCs *in vitro* have attracted wide interests of researchers. In addition, the distinct differentiation of NSCs has also raised the possibility that it may provide a novel source of neural cells for nervous tissue replacement or repair after injury [1, 2].

Previous studies have reported that the behavior of NSCs was regulated by both intrinsic characteristics and extrinsic signals originating from the external environment. It was identified that signals from cell-cell contact can also regulate the multi-potential of NSCs. For example, protoplasmic astrocytes and amnionic cells

can promote NSCs differentiating into neurons under co-culture conditions. Alternatively, epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) have been shown to induce the proliferation of NSCs in culture [3, 4]. In addition, neurotrophic factors such as neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF) have also been reported to be important factors determining the sub-type of differentiating NSCs [5-8].

Retinoic acid (RA) is a key regulator during embryonic patterning and development. Effective effect of RA is dependent on certain concentration in medium, among which, over 1500 nM RA in cultured medium will lead to the death of embryonic cells. It has been reported that during the development of nervous system, neurons can acquire axonal, dendritic polarity and form functional synapses through supplementing 500 nM RA [9, 10]. However, the same

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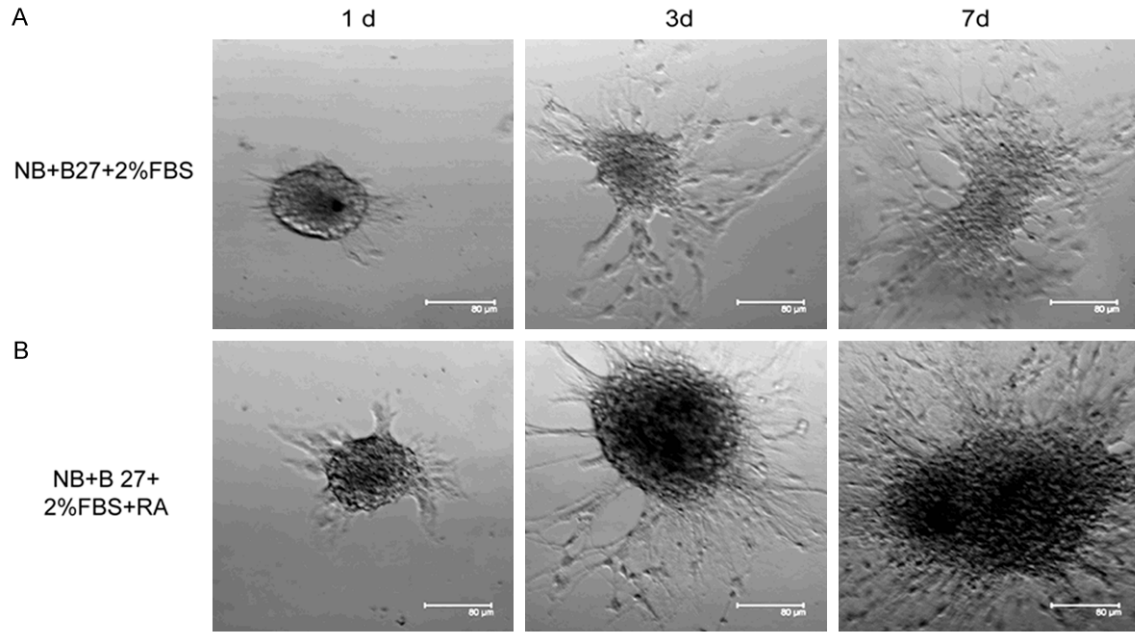


Figure 1. The growth and differentiation of NSCs cultured in different media at 1, 3, 7 days *in vitro*. A. Growth and differentiation of NSCs cultured in NB+B27+2% FBS medium at 1, 3 and 7 days. B. Growth and differentiation of NSCs cultured in NB+B27+2% FBS+500 nM medium at 1, 3 and 7 days. (Scale bar=80 μm).

concentration RA on the growth and function of differentiated neurons from NSCs of spinal cord and its potential mechanism is not clear. In this manuscript, the effects and mechanism of 500 nM RA on differentiation and synapses growth of NSC from embryonic spinal cord were investigated.

Materials and methods

Cell preparation

All experiments were approved by the animal care and experimental committee of the Third Medical Military University. Primary culture of NSCs was prepared from embryos (E14) of Wistar rats as described previously [11]. Briefly, the spinal cord tissue was rapidly dissected and placed into 3 ml tubes containing 0.25% trypsin. The tissue was then mechanically dissociated into single-cell suspension. Cell number and viability were assessed by staining with 0.4% trypan blue. Single-cell suspension was then transferred to growth medium consisting of NB+2% B27 supplemented with 20 ng/ml human recombinant basic fibroblast growth factor (bFGF, Gibco Invitrogen, USA), 20 ng/ml of epidermal growth factor (EGF, Gibco Invitrogen, USA) at 1×10^6 cells/ml. The cells were plated into culture flasks and maintained

under a humidified atmosphere of 5% CO₂ in air at 37°C. After 5 days *in vitro*, the neurospheres were dissociated into single-cell suspension and seeded onto 96-well plates at 1-2 cells per well. The neurosphere subcultures were digested and another passage was performed as before. The cell passage protocol was performed every 6 days to obtain neurospheres originating from a single primary cell. Secondary or tertiary neurospheres were used for subsequent experiments.

To examine the effect of RA, NSCs were divided into two groups: NSCs cultured in NB+2% B27+2% FBS medium during differentiation were regarded as control group. In RA group, the NSCs were maintained in NB+2% B27+2% FBS+500 nM RA medium during differentiation with medium exchanged every 3 days. On 7 days post-differentiation, the differentiated cells from NSCs in two groups were fixed and immunochemically stained with specific markers of neuron and astrocyte.

Immunocytochemical identification of differentiated neural cells

The differentiated cells from NSCs were characterized by certain antibodies, including rabbit anti-rat glial fibrillary acidic protein (GFAP; IgG,

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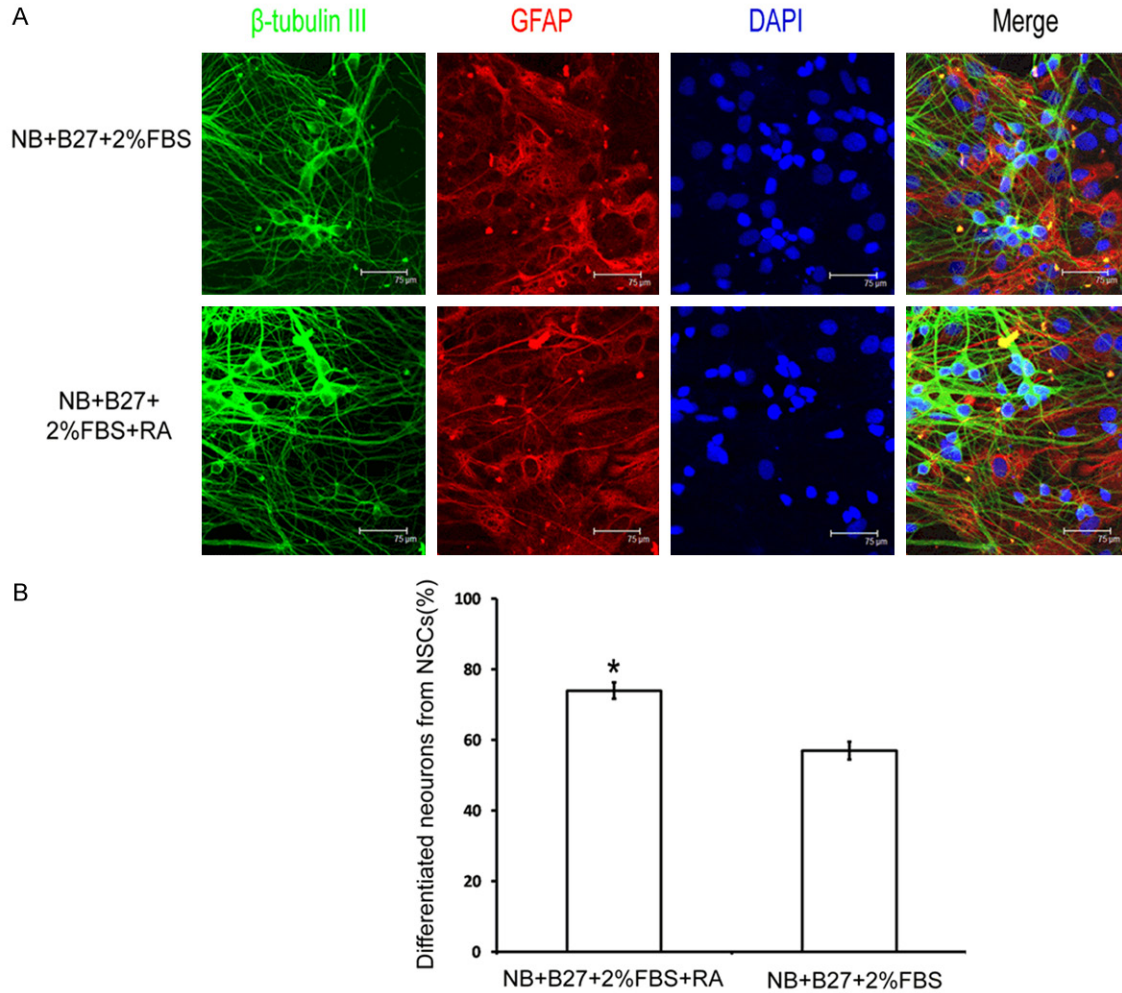


Figure 2. Identification and quantification of differentiated neural cells cultured in different media 7 days in vitro. A. Immunocytochemical staining of differentiated neural cells in two culture media. β -tubulin III staining (green) indicates neurons; GFAP staining (red) indicates astrocytes. The nuclei were counterstained with DAPI (blue). (Scale bar=75 μ m). B. Quantification of differentiated neurons in two culture media. *P<0.05 indicates statistical significance compared with the NB+B27+2% FBS group.

1:400; Sigma-Aldrich, USA) which is a specific marker for astrocyte, and the specific neuronal cell marker: mouse anti-rat β -tubulin III antibodies (1:800; IgG, Sigma-Aldrich, USA). Typical immunocytochemical procedures were applied for identification the differentiated cells [7, 8]. Briefly, the cells were fixed with 4% paraformaldehyde for 30 min in PBS, then permeabilized with 0.3% Triton X-100 for 20 min, and blocked with 5% normal goat serum. Subsequently, the cells were incubated with the above two primary antibodies overnight at 4°C, then incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibodies (1:100;

Chemicon, MA, USA) for 1 h at 37°C. Finally, the cells were counterstained with 300 nM of 4-6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich) for 3 min. Labeled cells were scanned with a Leica confocal microscope (SP-2, Leica, Germany). For negative controls, the cells were processed by the same immunofluorescent staining technique, but with omission of the primary antibodies.

Western blotting analysis

Total cell lysates were prepared by re-suspending 1×10^6 cells in 100 μ l Gel Sample Buffer (62.5 mM Tris-HCl, 10% glycerol, 5% 2-mercaptoethanol, 2% SDS and 0.025% bromophenol

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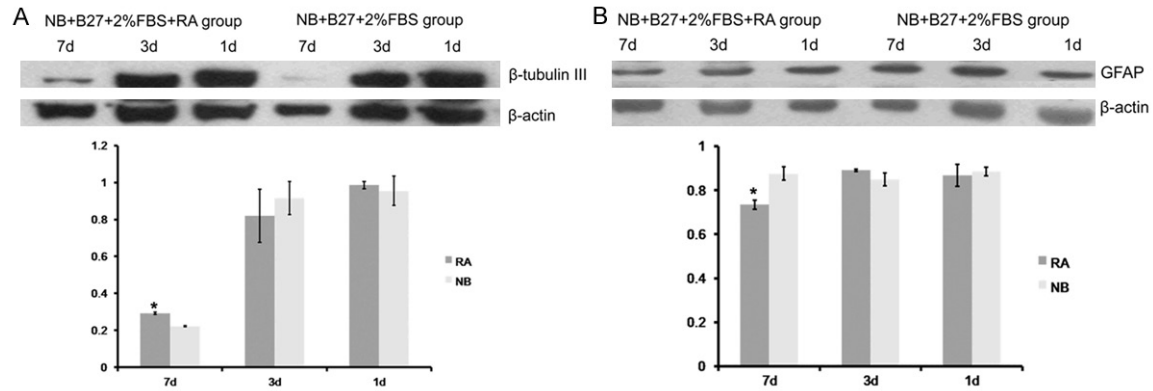


Figure 3. Western blotting analysis of β -tubulin III and GFAP protein in differentiated cells cultured with different media at 1, 3, 7 days in vitro. A. Western blotting showing the relative amount of β -tubulin III protein in two groups. B. Western blotting showing the relative amount of GFAP protein in two groups. β -actin was used to control for loading. Data represent mean \pm SD of three independent experiments. * $P < 0.05$ indicates statistical significance compared with the NB+B27+2% FBS group.

blue) and boiled for 5 min. Protein samples were run on polyacrylamide resolving gel (10%) and transferred onto a nitrocellulose membrane by electro-blotting at a constant current of 250 mA. Immunoblotting was performed by incubating the membrane overnight (4°C) with the primary antibodies: mouse anti-rat β -tubulin III (1:1000; Sigma-Aldrich), rabbit anti-rat GFAP (1:400; Sigma-Aldrich) respectively and detected using HRP-conjugated secondary antibody. Protein bands were visualized by an enhanced chemical luminescent detection system (Millipore) [9]. The data were normalized by running parallel Western blots with β -actin as an internal control. The optical density was quantified by the Image-Pro Plus 6.0 software. Separate experiments were conducted three times.

Calcium activity in differentiated neurons

The differentiated neural cells in two groups were incubated with 5 nM Fura-3-AM in Ca^{2+} -free D-Hanks medium for 30 minutes. After washed with 0.1 M PBS for three times, the medium was replaced with NB+2% B27 medium again. Then the differentiated neurons were observed timely with laser confocal scanning microscope at xyt model to examine the changes of free Ca^{2+} concentration in differentiated neurons stimulated by 1000 nM glutamine [8].

Statistical analysis

The percentage of positive cells in relation to the total cells was determined in 5 random fields under a 40 \times objective for each group in

five independent experiments. All data were presented as mean \pm SD. Statistical analysis of data was performed by a one-way analysis of variance (ANOVA). $P < 0.05$ was considered to be statistically significant.

Results

Differentiation of NSCs under different cultured conditions

On the second day after plating, the NSCs cultured in RA medium began to adhere to the bottom of culture dishes and grew out thin and short cytodendrites. After three days, the differentiated cells that migrated away from the spheres exhibited morphological characteristics of neurons, including spherical and refractile cell bodies with neurite-like processes (**Figure 1**). Seven days after differentiation, a large percent of cells had thin and long cytodendrites and the cytodendrites began to form connections with each other. In fact, these cells were β -tubulin III immuno-reactive positive neurons and were more abundant in RA culture medium than those in the control media.

To confirm differentiated cells from NSCs in the two cultured conditions, specific markers for astrocyte and neuron were applied for immuno-fluorescent staining (**Figure 2A**). The results indicated that 7 days after differentiation, 74% \pm 2.37 of differentiated cells in RA culture media expressed the neuronal specific marker β -tubulin III, which was significantly greater

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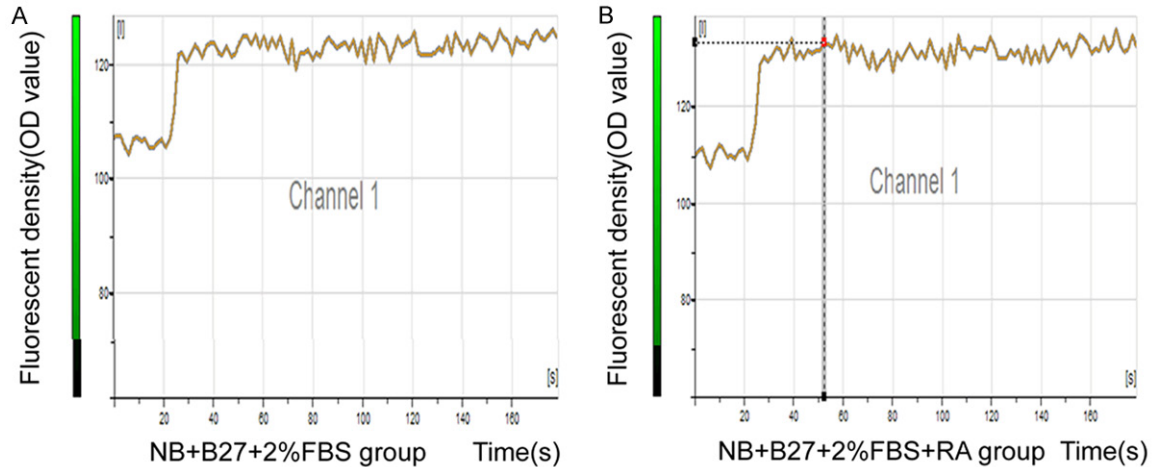


Figure 4. The changes of free calcium ions in cytoplasm of differentiated neurons after stimulated by glutamine. A. The curve of free calcium concentration in neurons differentiated from NSCs cultured in NB+B27+2% FBS medium. B. The curve of free calcium concentration in neurons differentiated from NSCs cultured in NB+B27+2% FBS+500 nM RA medium. Note: The line represents continuous change of free calcium ions concentration in cytoplasm of one differentiated neurons from NSCs after stimulation. Representative results of n=3 experiments.

than those in the control media ($57\% \pm 2.49$ β -tubulin III positive cells) (**Figure 2B**).

Moreover, western blot analysis confirmed β -tubulin III expression in differentiated neuron of the two media conditions. During the differentiating process, the level of β -tubulin III expression in RA medium was higher than those in the control medium at 7 days after differentiation. On the contrary, the quantity of GFAP in RA group was apparently less than that in the control group at corresponding time-points (**Figure 3**).

Calcium activity in differentiated neurons in two groups respectively

After adding 1000 nM glutamine to the differentiated cells in two cultured conditions, the Ca^{2+} outside the differentiated neurons flew quickly into the cytoplasm. The concentration of free calcium which indicated as fluorescent density in cytoplasm significantly increased and maintained at relatively high levels as shown in **Figure 4**. However, the concentration of free calcium inside the differentiated neurons from NSCs in two groups had no significant difference after stimulation.

Discussion

Because NSCs have the ability to self-renew and differentiate into neurons and glial cells

(astrocytes and oligodendrocytes), they were regarded as an ideal candidate for the developing treatments of central nervous system injury, such as stroke, neurodegenerative diseases, spinal cord and traumatic brain injury [1, 12, 13].

Less clear is the molecular mechanism that regulated the differentiation of NSCs under different conditions. Recent studies have demonstrated that, in addition to the intrinsic properties of stem cells, the extrinsic local microenvironment, including growth factors, cytokines and cell-cell contacts, plays an important role in determining the fate of stem cells [5, 14, 15].

Differentiation of NSCs into neurons *in vitro* has been examined through a variety of methods. A series of studies revealed that sequential treatment with NT-3, BDNF and Shh significantly promoted the neuronal differentiation from NSCs whether *in vitro* or *in vivo* after transplantation [5, 6, 14, 16]. These results suggest that the microenvironment play a prominent role for the induction of NSCs to differentiate into neurons.

RA is an important biologically active molecular that takes part in embryonic neurogenesis. During central nervous system development, RA plays an essential role in antero-posterior patterning of the hindbrain and spinal cord, dorsoventral patterning of spinal cord neurons

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and striatal neuron differentiation. Furthermore, RA can induce embryonic stem cell differentiation into GABAergic neurons and promote the neurogenesis of subventricular zone and striatal after stroke with special intervention. The effect of RA was dependent on the concentration in medium, among which 500 nM RA is suitable for the growth of embryonic cells *in vitro* [17-21]. These studies led us to investigate whether RA can enhance NSCs derived from embryonic spinal cord differentiating into functional neurons *in vitro*.

In this study, it was identified that exogenous supplementation of RA can promote the growth of cytodendrites of differentiated neuronal cells at early stage after differentiation and induce NSCs differentiating into more neurons compared to the control group in all. On the other hand, the astrocytes differentiated from NSCs were less in RA group than those in the control group. As indicated by immunocytochemical staining and Western blotting examination, it was indicated that RA have the ability to promote the growth of processes and distinct differentiation of NSCs into neurons from embryonic spinal cord. So in spinal cord injury, RA may be a candidate drugs for the treatment of spinal cord injury in the future.

As it has been reported that calcium in neural cells participated in the secretion and transmission of neurotransmitters, discharge of neurons and communication of organelle during the process of cell metabolism [22]. In this study, the free Ca^{2+} outside the cell membrane of differentiated neurons in two culture conditions can influx quickly into cytoplasm and maintained at high level after stimulated by glutamine. It was indicated that differentiated neurons from NSCs could receive outside stimulating signals and react with electrical activity. So the neurons differentiated supplemented with RA or not both have the characteristic of electrical activity of neuronal cells.

In all, it was suggested RA can promote the growth of cytodendrites of neuronal cells during the differentiating process and finally induce NSCs derived from embryonic spinal cord differentiating into functional neurons *in vitro*. Furthermore, the mechanism and signaling pathway of neuronal differentiation of NSCs in RA supplementation conditions are still to be further investigated.

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Disclosure of conflict of interest

None.

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References

- [1] Elisabeth D, Lukas S. Neural crest progenitors and stem cells: From early development to adulthood. *Dev Biol* 2012; 366: 83-95.
- [2] Fanie BH, Jonas F. Stem cells for spinal cord repair. *Cell Stem Cell* 2008; 3: 16-24.
- [3] Konagaya S, Kato K, Nakaji HT, Arima Y, Iwata H. Array-based functional screening of growth factors toward optimizing neural stem cell microenvironments. *Biomaterials* 2011; 32: 5015-5122.
- [4] Wang FW, Hao HB, Zhao SD, Zhang YM, Liu Q, Liu HJ, Liu SM, Yuan QH, Bing LJ, Ling EA, Hao AJ. Roles of activated astrocyte in neural stem cell proliferation and differentiation. *Stem Cell Res* 2011; 7: 41-53.
- [5] Moyse E, Segura S, Liard O, Mahaut S, Mechawar N. Microenvironmental determinants of adult neural stem cell proliferation and lineage commitment in the healthy and injured central nervous system. *Curr Stem Cell Res Ther* 2008; 3: 163-84.
- [6] Wang L, Kisaalita WS. Administration of BDNF/ginsenosides combination enhanced synaptic development in human neural stem cells. *J Neurosci Meth* 2011; 194: 274-282.
- [7] Liu Y, Liu RR, Wang L, Long ZY, Zeng L, Wu YM. The effect of different phenotype astrocytes on neural stem cells differentiation in co-culture. *Neurosci Lett* 2012; 508: 61-66.
- [8] Liu Y, Wang L, Long ZY, Zeng L, Wu YM. Protoplasmic astrocytes enhance the ability of neural stem cells to differentiate into neurons *in vitro*. *PLoS One* 2012; 7: e38234.
- [9] Christie VB, Maltman DJ, Henderson AP, Whiting A, Marder TB, Lako M, Przyborski SA. Retinoid supplementation of differentiating human neural progenitors and embryonic stem cells leads to enhanced neurogenesis *in vitro*. *J Neurosci Meth* 2010; 193: 239-245.

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- [10] Jennifer MP, Justin TW, Tim S, Jack MP. Retinoic acid and environmental alter subventricular zone and striatal neurogenesis after stroke. *Exp Neurol* 2008; 214: 125-134.
- [11] Liu XC, Zhu Y, Gao W. Isolation of neural stem cells from the spinal cords of low temperature preserved abortuses. *J Neurosci Meth* 2006; 157: 64-70.
- [12] Milet C, Monsoro-Burq AH. Neural crest induction at the neural plate border in vertebrates. *Dev Biol* 2012; 366: 22-33.
- [13] Yang K, Jung SL, Kim J, Lee YB, Shin H, Um SH, Kim JB, Park KI, Lee H, Cho SW. Polydopamine-mediated surface modification of scaffold materials for human neural stem cell engineering. *Biomaterials* 2012; 33: 6952-6964.
- [14] Oliver C, Hargus G, Deleidi M, Blak A, Osborn T, Marlow E, Lee K, Levy A, Perez-Torres E, Yow A, Isacson O. Differentiation of human ES and Parkinson's disease iPS cells into ventral mid-brain dopaminergic neurons requires a high activity form of SHH, FGF8a and specific regionalization by retinoic acid. *Mol Cellular Neurosci* 2010; 45: 258-266.
- [15] Tarasenko YI, Yu Y, Jordan PM, Bottenstein J, Wu P. Effect of growth factors on proliferation and phenotypic differentiation of human fetal neural stem cells. *J Neurosci Res* 2004; 78: 625-36.
- [16] Cheng XX, Wang YP, He Q, Qiu MS, Whittemore SR, Cao QL. Bone morphogenetic protein signaling and Olig1/2 interact to regulate the differentiation and maturation of adult oligodendrocyte precursor cells. *Stem cell* 2007; 25: 3204-3214.
- [17] Chen W, Jia W, Wang K, Zhou Q, Leng Y, Duan T, Kang J. Retinoic acid regulates germ cell differentiation in mouse embryonic stem cells through a Smad-dependent pathway. *Biochem Biophys Res Commun* 2012; 418: 571-577.
- [18] Kim M, Habiba A, Doherty JM, Mills JC, Mercer RW, Huettner JE. Regulation of mouse embryonic stem cell neural differentiation by retinoic acid. *Dev Biol* 2009; 328: 456-471.
- [19] Ostadsharif M, Ghaedi K, Nasr-Esfahani MH, Mojbafan M, Tanhaie S, Karbalaie K, Baharvand H. The expression of peroxisomal protein transcripts increased by retinoic acid during neural differentiation. *Differentiation* 2011; 81: 127-132.
- [20] Okada Y, Shimazaki T, Sobue G, Okano H. Retinoic acid concentration dependent acquisition of neural cell identity during in vitro differentiation of mouse embryonic stem cells. *Dev Biol* 2004; 275: 124-142.
- [21] Tonge PD, Andrews PW. Retinoic acid directs neuronal differentiation of human pluripotent stem cell lines in a non-cell-autonomous manner. *Differentiation* 2010; 80: 20-30.
- [22] Yi JH, Hazell AS. Excitotoxic mechanisms and the role of astrocytic glutamate transporters in traumatic brain injury. *Neurochem Int* 2006; 48: 394-403.