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Administration of BDNF/ginsenosides combination enhanced synaptic development in human neural stem cells

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ABSTRACT

Ginsenosides Rg1 and Rb1, major pharmacologically active ingredients from Ginseng, the root of Panax ginseng C.A. Meyer (Araliaceae), were applied in the differentiation media for human neural stem cells (hNSCs), together with brain-derived neurotrophic factor (BDNF), a commonly used compound for neural stem cell (NSC) differentiation. Cell locomotion and neurite extension were observed by time-lapse microscopy and analyzed by ImageJ software. The expression of synaptic formation was confirmed by immunostaining of synaptophysin (SYN) or/and the co-localization of synapsin I and microtubule associated protein-2 (MAP-2). Effects of cell density on neural differentiation were also examined. Results have shown that administration of BDNF/ginsenosides (Rg1 and Rb1) combination in differentiation medium promoted cell survival, enhanced neurite outgrowth and synaptic marker expression during differentiation. High cell density enhanced synaptic marker expression in BDNF/ginsenosides combination medium. In all, this study established a condition for hNSCs synaptic development in early differentiation, which is a crucial step in applying this cell line in neural network-based assay.

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1. Introduction

Neural stem cells (NSCs), which are derived from embryonic stem cells (ESCs) are partially differentiated, multipotent and can develop into any type of neural cells (e.g. neurons and astrocytes) *in vitro* (Zhang, 2006). Also, NSCs are capable of proliferating, thus providing an unlimited cell source. In pharmaceutical and biotechnology industries, human NSCs (hNSCs) are being considered as a promising cell source in preclinical studies (Irion et al., 2008). While in tissue engineering and regenerative medicine, these cells are actively being investigated for neurodegenerative disease therapy (Ogawa et al., 2009). There is interest in differentiating hNSCs to mature functional neural networks for preclinical studies of drugs against connectivity related targets (e.g. N-methyl-D-aspartic acid receptors (NMDARs)) (Meador-Woodruff et al., 2003; Wang and Kisaalita, 2010; Wang et al., submitted for publication).

NSC differentiation is regulated by various microenvironment cues, such as chemical and physical properties of extracellular matrix (Little et al., 2008), and functional neural network development from NSCs has been proven to be microenvironment dependent (Illes et al., 2009). Among these effective molecules that affect neuronal fate (Schaffer and Gage, 2004), brain-derived neurotrophic factor (BDNF) is an effective modulator of hNSC differentiation and neuronal cell survival (Poo, 2001). It plays an important role in the survival of differentiated neural stem cells through the MAPK/ERK-dependent and PI3K/Akt-dependent Bcl-2 up-regulation (Lim et al., 2008). Also, it has been proven to accelerate the maturation of the synaptic vesicle protein synapsin-1 at developing neuromuscular junctions in cell cultures (Poo, 2001). Thus, BDNF has been often added in neural stem cell differentiation media (Johnson et al., 2007).

Ginseng, the root of Panax ginseng C.A. Meyer (Araliaceae), has been extensively used in traditional oriental medicine for over 2000 years. *In vivo* and *in vitro* studies have shown its beneficial effects in cardiovascular diseases, cancer and immune deficiency (Cheng et al., 2005). A recent study has suggested that some of ginseng's active ingredients also exert beneficial effects on aging and neurodegenerative diseases (Radad et al., 2006). Ginsenosides such as Rb1 (MW 1109.29 Da) and Rg1 (MW 801.02 Da) are the major pharmacologically active ingredients of ginseng and their anti-aging and anti-neurodegeneration effects have been well proven (Cheng et al., 2005). Cell-based studies have shown that ginsenosides promoted NSC proliferation *in vitro* and enhanced cell survival (Shen and Zhang, 2004). Mechanisms may involve decreasing NO content and NOS activity, reducing intracellular calcium concentration, by up-regulating Hes1 expression, enhancing superoxide dismutase (SOD) activity and enhancing the ratio of Bcl-2 to Bax protein and inhibiting activation of caspase-3 (Cheng et al., 2005; Zhuang et al., 2009). Ginsenosides Rg1 and Rb1 also promoted neurite outgrowth in PC12 cells (Rudakewich et al., 2001), enhanced astrocyte

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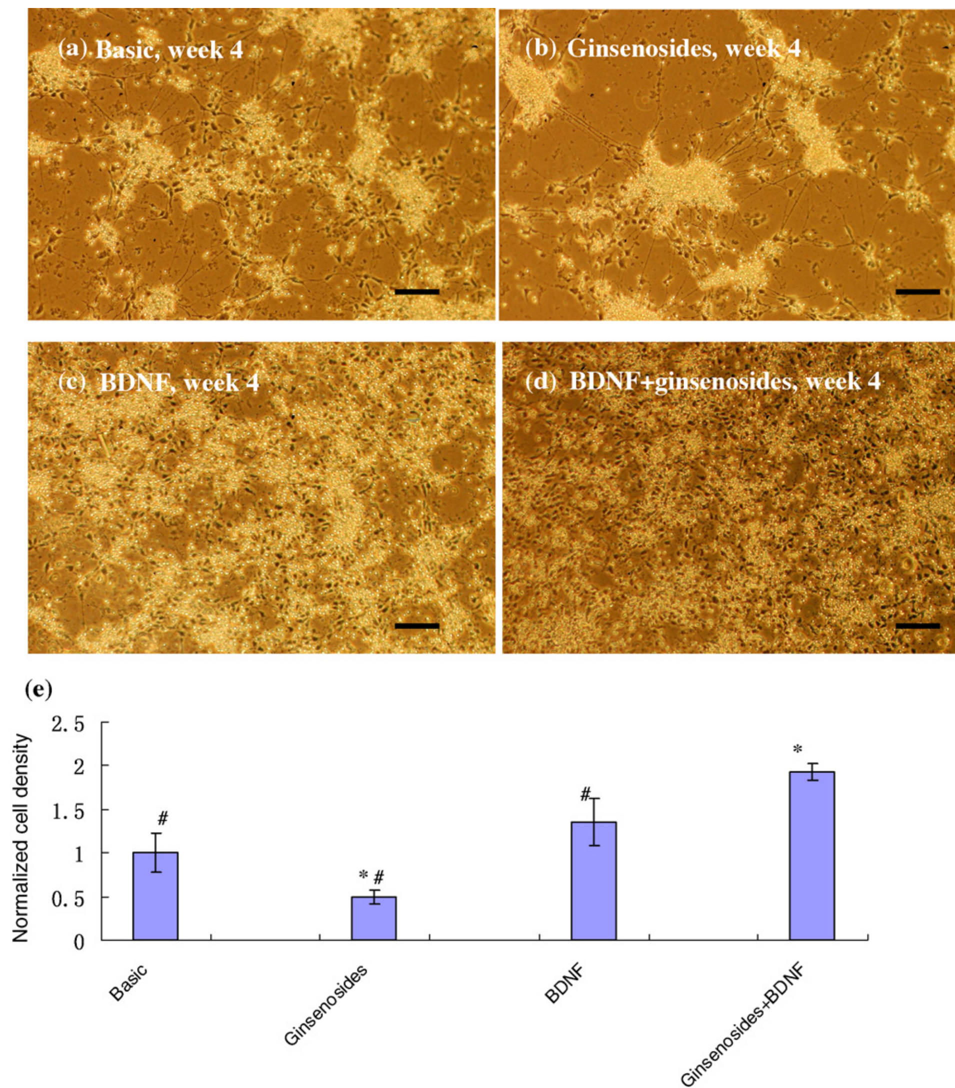


Fig. 1. (a–d) Phase contrast images of hNSCs at week 4 in differentiation in basic differentiation media (a), basic media with ginsenosides Rg1 (5 μM) and Rb1 (5 μM) (b), basic media with BDNF (10 ng/ml) (c), basic media with combination of BDNF (10 ng/ml) and ginsenosides Rg1 (5 μM) and Rb1 (5 μM) (d). Scale bar = 100 μm. (e) Cell densities (mean ± S.D.) were normalized by dividing with the mean density from the basic differentiation media group. $^{*}P < 0.05$, compared to the group with basic differentiation media; $^{\#}P < 0.05$, compared to the group with the combinational differentiation media.

differentiation from NSCs (Shi et al., 2005), increased neurotransmitter release (Xue et al., 2006), and increased synapse number and the density of synaptophysin, which is the morphological basis for explaining Rb1 and Rg1 induced facilitation of learning and memory (Mook-Jung et al., 2001).

In this study, we explored the effects of BDNF/ginsenosides (Rg1 and Rb1) combination on hNSC differentiation. Based on previous studies of BDNF and ginsenosides for neural cell survival and differentiation, it was expected that the combination could enhance synaptic development in hNSC differentiation than BDNF or ginsenosides, separately. We also studied the effects of cell density on hNSC differentiation with respect to synaptic formation. The goal was to establish a hNSC differentiation condition for neural network connectivity development, reflected by synaptic formation.

2. Materials and methods

2.1. Cell line and cell culture

hNSCs, purchased from Millipore (ENStem-A^{TM}, Billerica, MA), were derived from WA09 human ESCs using methods previously described (Shin et al., 2005). Cells were maintained as previously described (Wang and Kisaalita, 2010). Briefly, cells were cultured in 35 mm Petri dishes with 2 ml growth medium in a 5% CO_2 humidified atmosphere at 37 °C. ENStem-A^{TM} expansion media supplemented with penicillin/streptomycin, L-glutamine and basic fibroblast growth factor (bFGF) was used. The medium was half changed every other day. Cells were passaged by mechanically pipetting when cells reached 90–100% confluence and around 1.2×10^6 cells were seeded into each new dish. For cell differentiation, expansion media was replaced with differentiation media. Compared with expansion media, bFGF was withdrawn in basic differentiation media (Yan et al., 2005). Ginsenosides Rg1 (5 μM) and Rb1 (5 μM) (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) and BDNF (10 ng/ml, Invitrogen, Carlsbad, CA) were added in basic differentiation media. The concentrations were decided based on previous studies (Johnson et al., 2007; Shi et al., 2005). There were four differentiation conditions: (1) basic differentiation media; (2) ginsenosides Rg1 (5 μM) and Rb1 (5 μM) in basic differentiation media; (3) BDNF (10 ng/ml) in basic differentiation media; (4) combination of BDNF (10 ng/ml) and ginsenosides Rg1 (5 μM) and Rb1 (5 μM) in basic differenti-

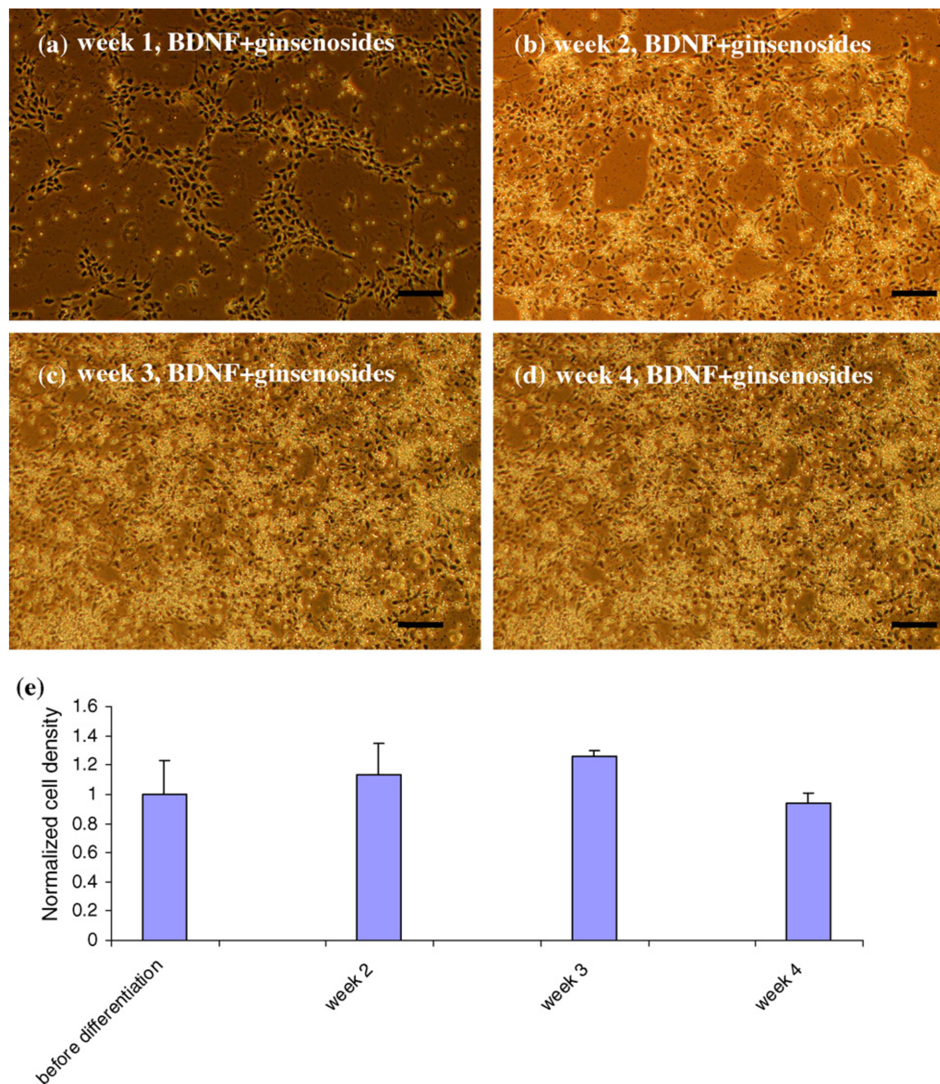


Fig. 2. Phase contrast images of neural stem cells before differentiation (a), during differentiation at week 2 (b), week 3 (c), and week 4 (d). Differentiation media contained BDNF (10 ng/ml) and ginsenosides Rg1 (5 μM) and Rb1 (5 μM). Scale bar = 100 μm. (e) Cell densities (mean ± S.D.) were normalized by dividing with the mean density from the group before differentiation. Cell densities did not change significantly during differentiation ($P > 0.05$).

ation media. The differentiation medium was half changed every other day. Phase contrast images were taken at different time points (before differentiation, week 2, week 3, and week 4 in differentiation) for analysis ($n = 3$). According to our previous studies on this cell line (data not shown), dead cells are bright and form clusters; while live cells attach to the substrates and are darker. Thus, live cells were distinguishable and were counted. Cell densities were calculated (mean ± S.D.) and normalized by dividing with the mean density from basic differentiation media group. ANOVA was used for statistical comparisons.

2.2. Cell locomotion observation by time-lapse recording

We used time-lapse video microscopy as previously described (Wang et al., submitted for publication) to study neural stem cell locomotion before differentiation and during differentiation. Briefly, cells were differentiated with basic differentiation media or combined differentiation media for 3 weeks, then cells were plated onto coverslips and their locomotion was recorded for 10 h. The WaferGen Smart Slide-100 system (WaferGen, Fremont, CA, USA) was used to keep the cells in proper conditions while recording. This small environmental chamber is based on a 6-well dish. It uses heated glass to maintain temperature even at the spot being observed, and regulates CO_2 flow. Phase contrast images were taken every minute using Leica DFC300 FX camera mounted on a Leica Z16 APO A microscope. Image sequences and trajectory plots were generated by ImageJ software (NIH). Manual analysis of cell locomotion speed (average speed within the first 10 h after plating) and neurite outgrowth (at the end of 10 h recording) was carried out according to methods published by Chen et al. (2009) and Mitchell et al. (2007), respectively. Data for cell movement before and during differentiation were compared by Student t-test.

2.3. Immunostaining for synaptic markers

We used 2 sets of staining to confirm the expression of synaptic markers: staining for the expression of synaptophysin (SYN) and the co-localization of synapsin I and microtubule associated protein-2 (MAP-2). Protocols from Aruna Biomedical Inc. (Athens, GA) were followed. Briefly, cells were rinsed twice with phosphate-buffered saline (PBS), fixed with 2% paraformaldehye

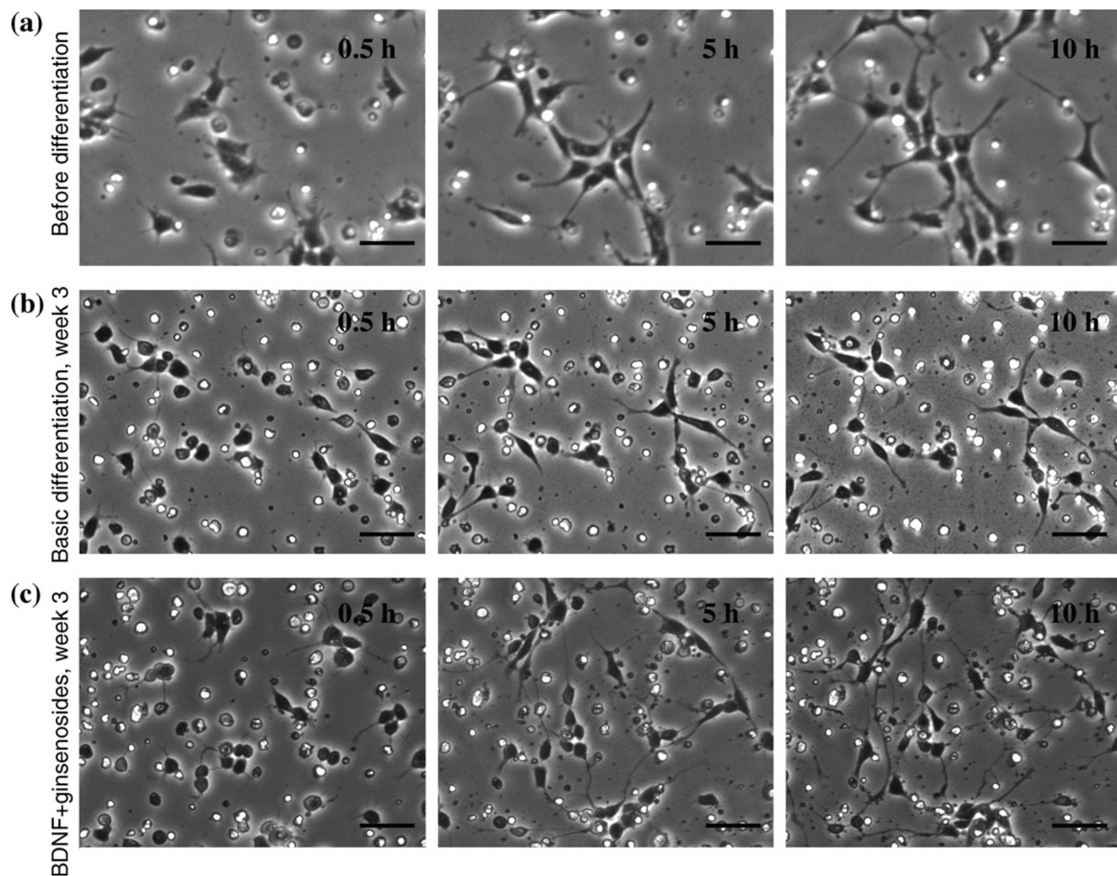


Fig. 3. Image sequences of cell locomotion before differentiation (a) and at week 3 in differentiation (b and c). Images showed cells on 2D flat surface after plating at 0.5 h, 5 h and 10 h. (a) Cells were kept in proliferation media. (b) Cells were differentiated in basic differentiation media. (c) Cells were differentiated in combinational differentiation media (BDNF (10 ng/ml) and ginsenosides Rg1 (5 μM) and Rb1 (5 μM)). Before differentiation, cells interacted with other cells, moved and formed cellular clusters (a). In differentiation, cell bodies moved in a confined area, and extended out neurites to get connection with other cells (b and c). Scale bar = 50 μm.

in PBS (15 min), permeablized with 0.5% Tween 20 (3 times, 5 min each), blocked for 45 min in blocking buffer, which is comprised of 6% from the animal that the second antibodies were produced in. Cells were exposed to a mixture of rabbit polyclonal anti-synapsin I (1:500) (Chemicon, Temecula, CA) and monoclonal anti-MAP2 (1:500) (Sigma, St. Louis, MO), or samples were incubated with polyclonal rabbit anti-human SYN (1:200) (DakoCytomation, Denmark) overnight at 4 °C. After exposure to primary antibodies, samples were washed with PBS (4 times, 5 min each), and incubated for 1 h at room temperature with blocking buffer containing a mixture of rhodamine-conjugated goat anti-rabbit IgG (1:500) and fluorescein-conjugated donkey anti-mouse IgG (1:500) (Jackson Immunological Research, West Grove, PA), or rhodamine-conjugated goat anti-rabbit IgG (1:500) (Jackson ImmunoResearch Laboratories, West Grove, PA). To capture fluorescence images of cells stained for SYN or synapsin I, cells were scanned with the Green HeNe laser and the resulting fluorescent signals were captured through a 565 nm long-pass filter (BA1, Nikon, Melville, NY) by a photomultiplier detector. Fluorescence images of cells stained for MAP-2 were viewed and captured with a B-2E/C FITC filter block (Nikon, Melville, NY, USA), which has an excitation bandwidth of 465–495 nm and a filter pass range of 515–555 nm. Fluorescence intensity of neurites was measured with Simple PCI image software (Compix Inc., Cranberry Township, PA). Fluorescence intensities (mean ± S.D.) were normalized by dividing with the mean value under basic condition at each week in differentiation. ANOVA was used for statistical comparisons.

3. Results and discussions

3.1. Combination of ginsenosides with BDNF promotes hNSC survival during differentiation

Cells were kept in differentiation media for 4 weeks, and Fig. 1(a)–(d) presents phase contrast images of hNSCs at week 4 in differentiation under different conditions. Dead cells, which were brighter than the live cells attaching on the substrates, were observed in all these samples. There were more dead cells in groups under basic differentiation (a) or ginsenosides-induced differentiation (b) than in groups under BDNF-induced differentiation (c) or BDNF/ginsenosides combination-induced differentiation (d). Normalized cell densities are presented in Fig. 1(e). Administration of BDNF or ginsenosides did not significantly increase cell survival at week 4 in differentiation. There were significantly more cells surviving in groups under BDNF/ginsenosides combination-induced differentiation ($P < 0.05$). By examining hNSCs in combination group at week 2, week 3 and week 4 in differentiation (Fig. 2(a)–(d)), it is interesting to notice that there was no significant difference among the cell densities during differentiation ($P > 0.05$). At week 3 and week 4 in differentiation, some dead cells were observed; however, there was no significant decrease in cell density. As shown in other studies (Saha et al., 2008), cell numbers decrease during differentiation, indicating that not all these cells could survive and differentiate to neural cells. Our results indicated that BDNF and ginsenosides could work synergically to maintain cell viability during differentiation. BDNF plays an important role in the survival of

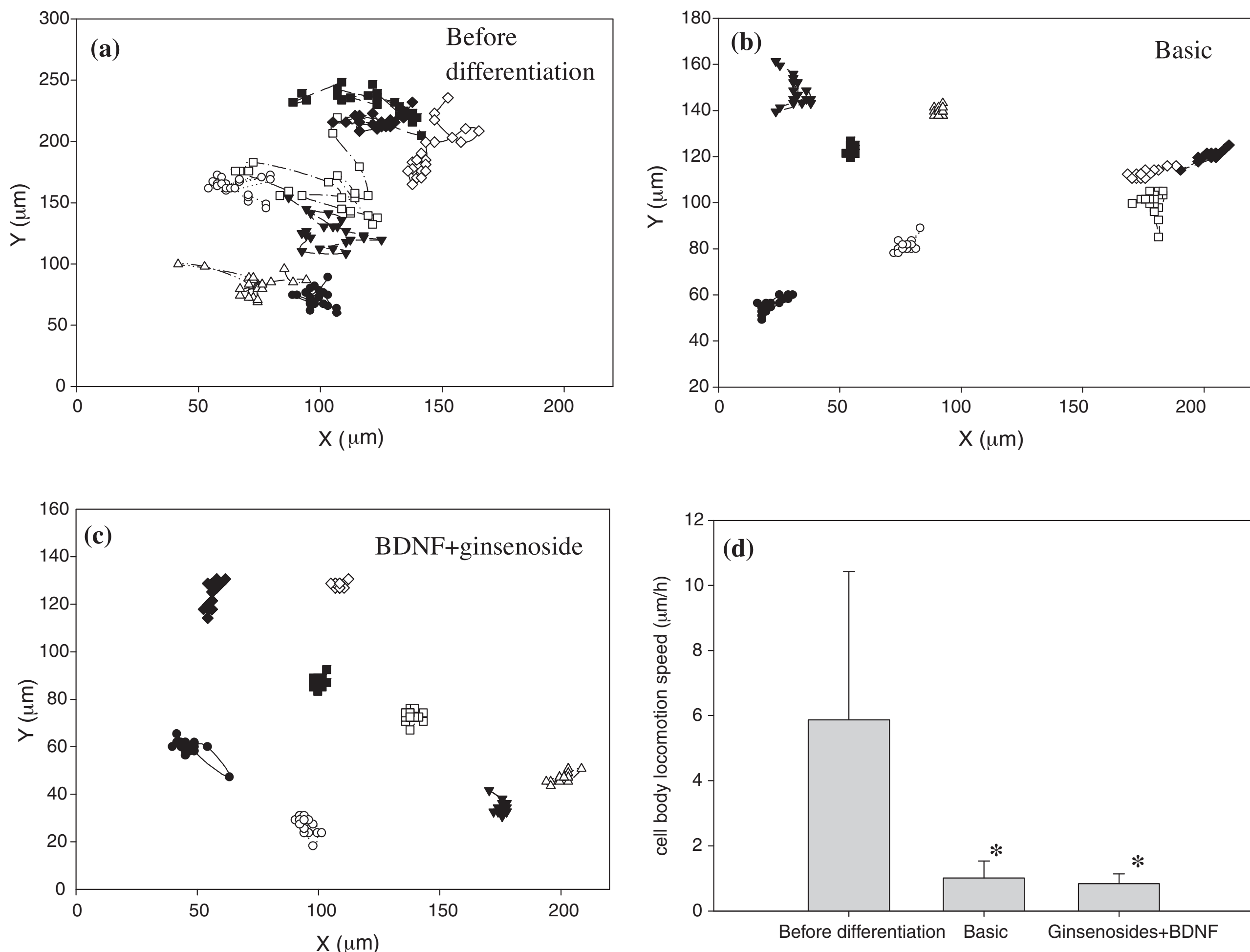


Fig. 4. Trajectory plots for hNSCs before differentiation (a) and at week 3 in differentiation (b and c). Cells were plated and observed for 10 h. For each group, 8 cells are presented. (a) Cells were kept in proliferation media. (b) Cells were differentiated in basic differentiation media. (c) Cells were differentiated in combinational differentiation media (BDNF (10 ng/ml) and ginsenosides Rg1 (5 μM) and Rb1 (5 μM)). (d) Cell body locomotion speed analysis. Cells before differentiation moved faster than cells in differentiation ($P < 0.05$).

differentiated neural stem cells through the MAPK/ERK-dependent and PI3K/Akt-dependent Bcl-2 up-regulation (Lim et al., 2008). And ginsenosides promoted neural stem cell proliferation *in vitro* and enhanced cell survival (Shen and Zhang, 2004) through mechanisms that may involve decreasing NO content and NOS activity, reducing intracellular calcium concentration, up-regulating Hes1 expression, enhancing superoxide dismutase (SOD) activity, and enhancing the ratio of Bcl-2 to Bax protein (Cheng et al., 2005; Zhuang et al., 2009). In the following studies, combination of BDNF and ginsenosides (Rg1 and Rg1) was applied in differentiation media.

3.2. *Combination of ginsenosides and BDNF promotes hNSC neurite outgrowth during differentiation*

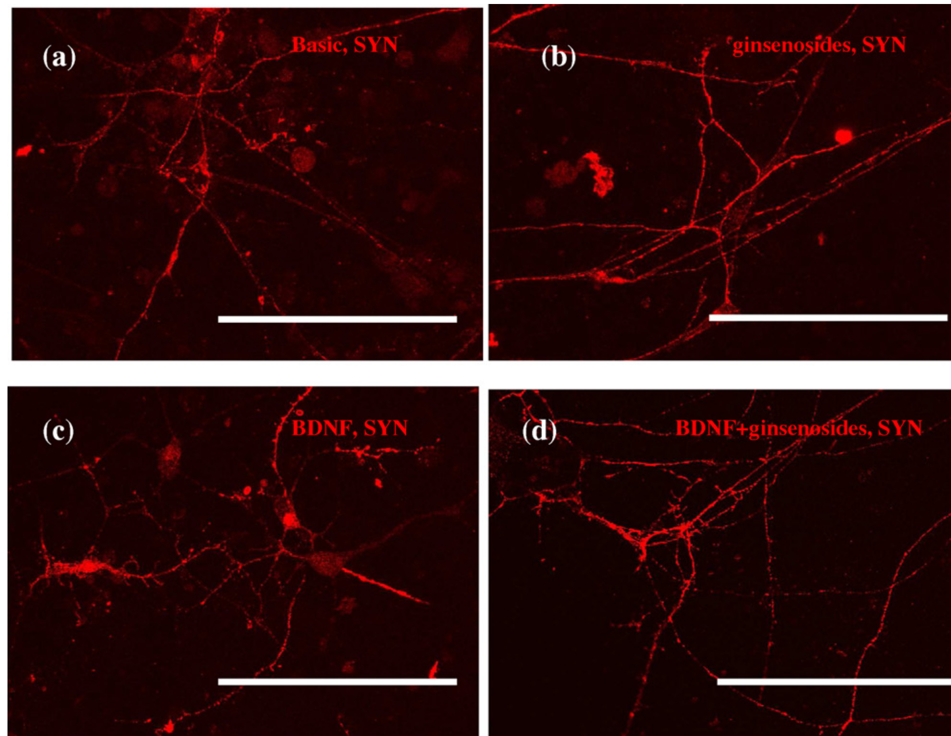
Time-lapse recording was used to study hNSC locomotion before and after differentiation. To explore the effects of combinational differentiation media on hNSC differentiation with respect to neurite outgrow, cells in basic differentiation media were used as a control for cells in combinational differentiation media. Figs. 3 and 4 show image sequences and trajectory plots, respectively, for hNSCs before differentiation and at week 3 in differentiation. Before differentiation, cells moved randomly at a speed of 5.86 ± 4.57 μm/h (Fig. 4(d)) and interacted with other cells and formed cellular clusters (Figs. 3(a) and 4(a)). Cells moved at significantly lower speeds of 1.01 ± 0.53 μm/h and 0.83 ± 0.31 μm/h in basic and combination differentiation media, respectively ($P < 0.05$). Our results confirmed the active hNSC locomotion before differentiation. hNSC migration *in vivo* plays a crucial role in the early phase of histogenesis in the embryonic striatum (Hamasaki et al., 2003). Recent studies have shown NSC migration and invasion in 3D tumor cell aggregates *in vitro* (Heese et al., 2005). Cell–cell contact, as well as extracellular matrix (ECM) components and soluble proteins secreted into microenvironment by intermediate or final target cells, underlie the mechanisms for hNSC locomotion both *in vivo* and *in vitro* (Hamasaki et al., 2003; Heese et al., 2005). In our study, active hNSC locomotion may be driven by cell–cell contact to form connection with other cells (Wang et al., submitted for publication).

During the recording, differentiated cells were observed to connect to each other. Differentiated cells also formed cellular clusters, and neurite extensions from cellular clusters were observed (Figs. 3(b) and (c) and 4(b) and (c)). Table 1 presents the quantitative analysis of neurite outgrowth for cells in basic and combinational differentiation media at week 3. There were more cells growing neurites in combinational differentiation media, and these neurites were longer than those in basic differentiation media ($P < 0.05$). Neurite growth is considered a very import event in neuronal development, synapse formation, and neural regeneration. Pharmaceutical control of neurite growth (e.g. by nerve growth factor) is of great interest in clinical application (Mitchell et al., 2007).

Table 1
Summary of quantitative analysis of neurite outgrowth in neural stem cells at week 3 in differentiation in different media[a].

	Cells bearing branches (% of total cells)	Cells bearing neurites[b] (% of total cells)	Average neurite length per cell (μm)	Average length of longest neurites from each field (μm)
Basic differentiation media	49.2 ± 10.7[c]	11.3 ± 3.2	51.9 ± 12.1	60.8 ± 13.2
Combinational differentiation media	64.1 ± 11.0*	31.5 ± 13.6*	63.79 ± 18.6*	89.4 ± 16.3*

[a] 4 fields were selected from each group; total cell number from basic differentiation group was 107 (25, 30, 29, 23); total cell number from combinational differentiation group was 109 (25, 21, 27, 33).
[b] Neurite is defined as at least three times long of soma body.
[c] Mean ± standard deviation.
* $P < 0.05$, compared to basic differentiation group.



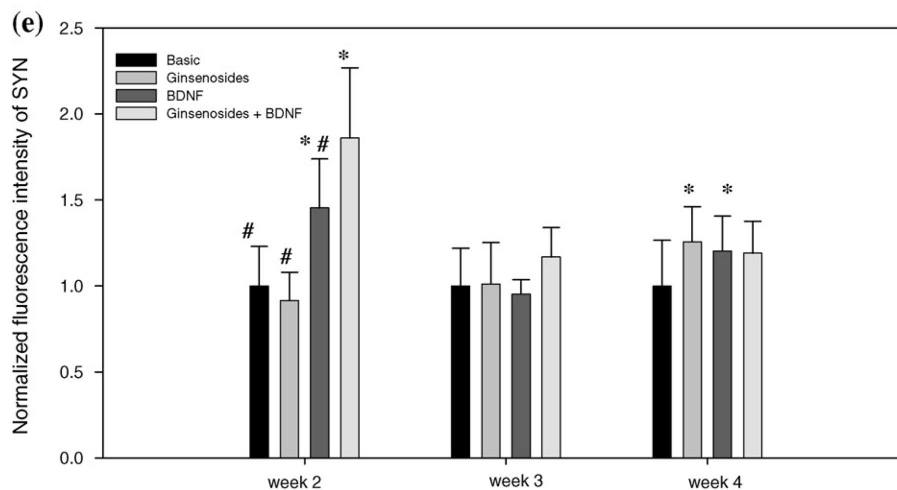


Fig. 5. (a–d) Confocal images of hNSCs at week 4 in differentiation. Cells were stained for SYN. Different molecules were added in basic differentiation media: (a) basic differentiation media; (b) ginsenosides Rg1 (5 μM) and Rb1 (5 μM); (c) BDNF (10 ng/ml); (d) BDNF (10 ng/ml) and ginsenosides Rg1 (5 μM) and Rb1 (5 μM). Scale bar = 100 μm. (e) Normalized fluorescence intensity of SYN staining for hNSCs in differentiation under different conditions. Basic: basic differentiation media; Ginsenosides: ginsenosides Rg1 (5 μM) and Rb1 (5 μM); BDNF: brain-derived neurotrophic factor (BDNF, 10 ng/ml); BDNF and ginsenosides: combination of BDNF (10 ng/ml) and ginsenosides Rg1 (5 μM) and Rb1 (5 μM). Fluorescence intensities (mean ± S.D.) were normalized by dividing with the mean value under basic condition at each week in differentiation. *$P < 0.05$, compared to the value of control group under basic condition at each week in differentiation. #$P < 0.05$, compared to the value of the combination group at each week in differentiation.

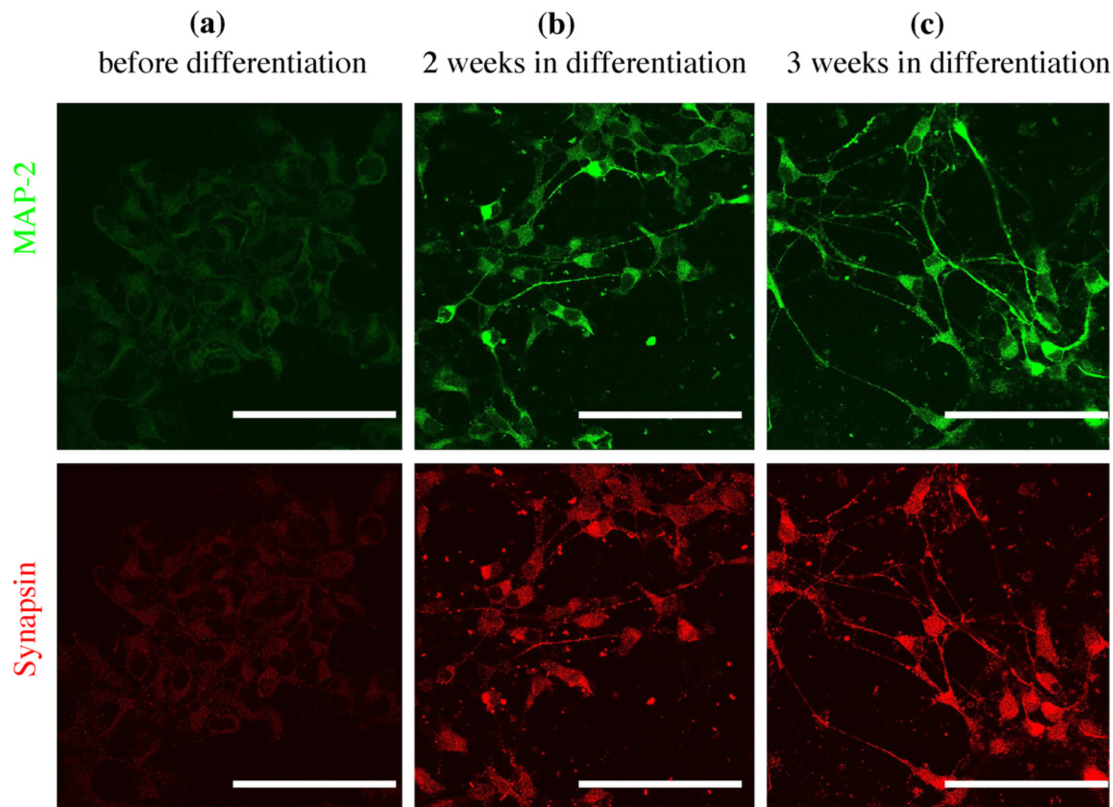


Fig. 6. Confocal images of hNSCs stained for MAP-2 (green) and synapsin (red) before differentiation (a), at week 2 (b) and week 3 (c) in differentiation. Differentiation media contained BDNF (10 ng/ml) and ginsenosides Rg1 (5 μM) and Rb1 (5 μM). Scale bar = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Shetty and Turner (1999) proved that BDNF could promote neurite outgrowth of NSCs. Our results were consistent with previous studies on effects of ginsenosides Rg1 and Rb1 on neurite outgrowth in PC12 cells (Rudakewich et al., 2001). In the presence of nerve growth factor, Rg1 and Rb1 stimulate neurite outgrowth in PC12 cells at day 8 in culture. Pathways or molecular mechanisms that regulate neurite outgrowth have been explored. It has been found that balance of MAP kinase and STAT3 signal transduction pathways regulated neurite growth in PC12 cells (Ihara et al., 1997), as well as protein kinase A-dependent molecular switch in synapsins in embryonic Xenopus spinal neurons (Kao et al., 2002). Based on these observations, it may be hypothesized that combinational differentiation media could promote hNSC differentiation with respect to synaptic formation. To further confirm the neural network formation, we examined the synaptic development of hNSCs in differentiation.

3.3. *The maximum expression of hNSC synaptic marker SYN occurred at week 2 in ginsenosides/BDNF combination-induced differentiation*

Fig. 5(a)–(d) presents the fluorescence images of cells stained for synaptophysin (SYN) at week 4 in differentiation, and staining was positive for these four differentiation conditions. At week 2, cells exhibited the strongest SYN signal under combination condition than other conditions (basic, ginsenosides, BDNF) (Fig. 5(e)). In the combinational group, SYN signal intensities did not change significantly with time in differentiation (data not shown). Under other conditions (basic, ginsenosides, BDNF), SYN signal intensities increased during differentiation (data not shown), and reached the same level as that of cells under combination condition (Fig. 5(e)). These results indicated that combination of ginsenosides and BDNF could promote synaptic marker SYN expression at an early time in differentiation. To further confirm this expression of synaptic connection, another set of immunostaining was used in this study: staining for the co-localization of synapsin I and MAP-2. Almost all the cells were positive for synaptic markers (MAP2, synapsin and SYN) on week 2 and week 3 in differentiation (Fig. 6). These results were consistent with previous reports. For example, BDNF has been proven to accelerate the maturation of the synaptic vesicle protein synapsin I at developing neuromuscular junctions in cell cultures (Poo, 2001). In other studies, ginsenosides were found to enhance astrocyte differentiation from neural stem cells (Shi et al., 2005), increase neurotransmitter release (Xue et al., 2006), and increase synapse number and the density of synaptophysin, which is the hypothesized basis of Rb1- and Rg1-induced facilitation of learning and memory (Mook-Jung et al., 2001).

3.4. *Cell density is a factor in BDNF/ginsenosides-mediated synaptic marker expression*

Cells were seeded at different densities (6×10^4 cells/cm^2, 10×10^4 cells/cm^2, 20×10^4 cells/cm^2) and were differentiated for 2 weeks by combination media containing BDNF (10 ng/ml) and ginsenosides Rg1 (5 μM) and Rb1 (5 μM). Fig. 7(a) presents phase contrast images of hNSCs after 2 weeks in differentiation. Synaptic markers (MAP-2 and SYN) were stained and signal intensity was measured and compared (Fig. 7(b) and (c)). Results showed that when cell density was too low ($<3 \times 10^4$ cells/cm^2), few cells survived in differentiation; as cell density increased from 6×10^4 cells/cm^2 to 20×10^4 cells/cm^2, both MAP and SYN expression increased significantly ($P < 0.05$). Other studies have shown that cell plating density is an important factor in neural differentiation. The development of resting membrane potentials in

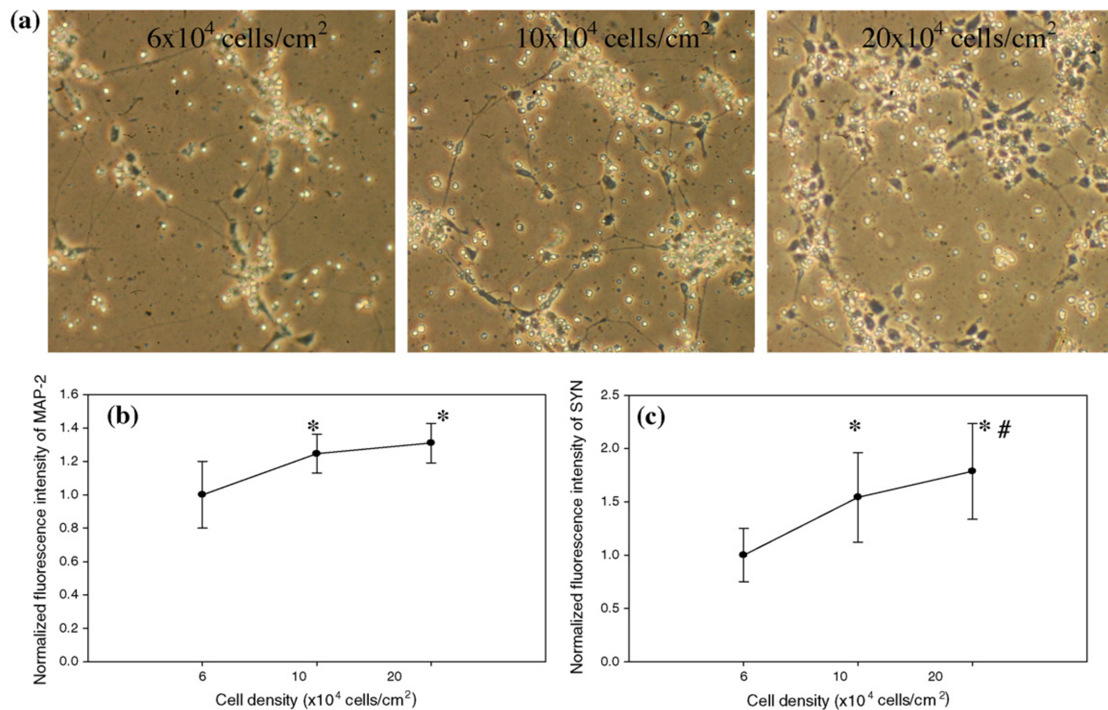


Fig. 7. Cell density effects on hNSC differentiation with respect to synaptic markers MAP-2 and SYN expression. Cells were seeded at different cell densities (left: 6×10^4 cells/cm^2, middle: 10×10^4 cells/cm^2, right: 20×10^4 cells/cm^2). Cells were differentiated for 2 weeks by combination media containing BDNF (10 ng/ml) and ginsenosides Rg1 (5 μM) and Rb1 (5 μM). (a) Phase contrast images of hNSCs. (b) and (c) Normalized fluorescence intensity of cells stained for MAP-2 (b) and SYN (c). Fluorescence intensities (mean ± S.D.) were measured and normalized by dividing with the mean value of cells seeded with the lowest cell density (6×10^4 cells/cm^2). $^*P < 0.05$, compared to the value at the cell density of 6×10^4 cells/cm^2; $^\#P < 0.05$, compared to the value at the cell density of 10×10^4 cells/cm^2.

differentiating murine neuroblastoma cells (NIE-115) was found be seeding cell density-dependent; lower cell density delayed the resting membrane potential development (Kisaalita and Bowen, 1997). Recent studies on stem cell differentiation have shown that seeding cell density is crucial in neuronal differentiation; when cell density was too low, cell viability was reduced (Lorincz, 2006). In order to attain high percentage of dopamine neuron population in mesencephalic precursor cultures, cultures needed to be seeded at high cell density (Ko et al., 2005). Our results are consistent with previous studies, confirming the seeding density effect on synaptic development in hNSC differentiation. Tsai and McKay (2000) reported that cell density effect on cortical stem cell fate determination depends on cell–cell contact and membrane-bound signals. Other studies have shown that cell density-mediated neural differentiation of stem cells was related to signaling pathways such as Rho kinases and β-Catenin signaling (Otero et al., 2004; Chang et al., 2010). However, the mechanisms underlying cell density-related synaptic development are still unknown.

4. Conclusion

In this study, we examined BDNF/ginsenosides (Rg1 and Rb1)-induced hNSC differentiation with respect to cell survival, cell dynamic locomotion, neurite outgrowth and synaptic development. Results have shown that administration of BDNF/ginsenosides combination promoted cell survival, enhanced neurite outgrowth, network formation and synaptic development during differentiation. Also, we examined cell density effects on hNSC differentiation and results indicated that cell density should be kept at certain level (such as 20×10^4 cells/cm^2) to maximize synaptic formation. Taken together, this study provided an effective protocol for hNSC differentiation with synapse formation, which is critical in neural-network based assay for drug discovery as well as in neuroregenerative medicine.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jneumeth.2010.10.025.

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