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journal homepage: <http://ees.elsevier.com/apjtm>Original research <http://dx.doi.org/10.1016/j.apjtm.2015.09.004>Effects of *Gastrodiae rhizoma* on proliferation and differentiation of human embryonic neural stem cellsSamrat Baral^{1,2,3,4}, Ramesh Pariyar^{2,4}, Chi-Su Yoon^{2,5}, Dong-Cheol Kim^{2,5}, Jong-Min Yun⁶, Seok O. Jang⁶, Sung Yeon Kim², Hyuncheol Oh^{2,3,4,5}, Youn-Chul Kim^{1,2,3,4,5}, Jungwon Seo^{1,2,3,4*}¹From the BK21 Plus Team, South Korea²Institute of Pharmaceutical Research and Development, College of Pharmacy, Wonkwang University, Iksan 570-749, South Korea³Professional Graduate School of Oriental Medicine, Wonkwang University, Iksan 570-749, South Korea⁴Hanbang Body-Fluid Research Center, Wonkwang University, Iksan 570-749, South Korea⁵Standardized Material Bank for New Botanical Drugs, College of Pharmacy, Wonkwang University, Iksan 570-749, South Korea⁶Department of Internal Medicine, College of Korean Medicine, Wonkwang University, Iksan 570-749, South Korea

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ABSTRACT

Objective: To investigate the effects of *Gastrodiae rhizoma*, a dried root of *Gastrodia elata* Blume, on proliferation and differentiation of human NSCs derived from embryonic stem cells.**Methods:** A 70% ethanol extract of *Gastrodiae rhizoma* (EEGR) was estimated with 4-hydroxybenzyl alcohol as a representative constituent by HPLC.**Results:** MTT assay showed that the treatment with EEGR increased the viability of NSCs in growth media. Compared to control, EEGR increased the number of dendrites and dendritic spines extended from a differentiated NSC. Whereas EEGR decreased the mRNA expression of *Nestin*, it increased that of *Tuj1* and *MAP2* in NSCs grown in differentiation media. Immunocytochemical analysis using confocal microscopy also revealed the increased expression of *MAP2* in dendrites of EEGR-treated NSCs. Furthermore, EEGR decreased mRNA expression of *Sox2* in NSCs grown even in growth media.**Conclusions:** In conclusion, our study demonstrates for the first time that EEGR induced proliferation and neuronal differentiation of NSCs, suggesting its potential benefits on NSC-based therapies and neuroregeneration in various neurodegenerative diseases and brain injuries.

1. Introduction

Neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, are caused by the loss of neurons and synapses in the brain and spinal cord. Adult CNS has only a limited ability to regenerate neurons that can be substituted for the injured neurons. The neural stem cells (NSCs) are multipotent cells to be

differentiated into many neuronal and glial cell lineages [1]. NSCs are assumed to be the promising resource for repairing the injured neurons in a cell-based therapy of various neurodegenerative disorders [2]. Indeed, the transplantation of NSCs derived from embryonic stem cells improved water maze performance in Alzheimer's disease model rats [3] and ameliorated circling behavior in the Huntington's disease model rats [4,5]. Despite the importance of NSC-based therapy, it is still difficult to promote NSC proliferation and to control neuronal differentiation. In addition, NSCs remain in an adult brain, mainly located in the subventricle ventricle zone [6] and hippocampal subgranular zone [7]. Although adult NSCs are restricted to a few and small neurogenic sites in the brain and remain relatively inactive or replace slowly the injured neurons, they have the potential to regenerate neurons and to repair the injured brain.

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Gastrodiae rhizoma, a dried rhizome of *Gastrodia elata* (*G. elata*) Blume, has been widely used as oriental herbal medicine for the treatment of various neurological symptoms, such as vertigo, hysteria, epilepsy, and paralysis [8]. The major constituents of this plant are 4-hydroxybenzyl alcohol, 4-hydroxybenzaldehyde, vanillyl alcohol, vanillin, and gastrodin [9]. *Gastrodiae rhizoma* extracts and its constituents have recently been evaluated for anticonvulsive activities [10], neuroprotective effects [11–14], antidepressant effects [15,16], and memory improvement [17,18]. Interestingly, there is a report showing that neuronal densities of hippocampal CA1 and CA3 increased in *Gastrodiae rhizoma*-administered rats after scopolamine treatment, compared to the control [19]. Furthermore, the administration of gastrodin, one of the major components, was reported to upregulate hippocampal NSCs proliferation measured by BrdU immunohistochemistry in depression rat model [20]. However, gastrodin failed to increase NSC viability in rat hippocampal NSC culture *in vitro*. In this study, we examined the effect of 70% ethanol extract of *Gastrodiae rhizoma* (EEGR) on the proliferation and differentiation of NSCs derived from human embryonic stem cells. Our result showed for the first time that EEGR promoted NSCs proliferation and their differentiation into neuronal cells, in particular, dendrite formation.

2. Materials and methods

2.1. Plant material and preparation of extract

The rhizomes of *G. elata* BL. (Orchidaceae) were purchased from Daehak Hanyakguk, Iksan, Korea in 2014, and authenticated by Prof. Youn-Chul Kim, Wonkwang University, Iksan, Korea. A voucher specimen (no. WP14-03) was deposited at the herbarium of the College of Pharmacy, Wonkwang University, Iksan, Korea. The dried rhizomes of *G. elata* (200 g) were grinded and extracted with 70% ethanol (800 mL) at Soxhlet extractor for 3 h. The solvent was removed under reduced pressure to obtain a 70% ethanol extract (27.91 g).

2.2. High-performance liquid chromatography (HPLC)

Chromatographic experiments were performed on a YL-9100 series HPLC instrument equipped with a sample injector and a photodiode array UV/Vis detector (PDA) (YoungLin, Korea). For all experiments, an SHISEIDO CAPCELL PAK C-18 column (4.6 nm × 250 nm; 5 μm, SHISEIDO CO., Tokyo, Japan) was used as the stationary phase, and the injection volume was 20 μL. Samples were prepared to contain 5 mg/mL concentration of EEGR or 1 mg/mL concentration of 4-hydroxybenzyl alcohol (Sigma–Aldrich). The mobile phase was composed of water (contain 0.1% formic acid) (A) and acetonitrile (B), with gradient system; 0–40 min linearly changed 10% B to 30% B, 40–50 min linearly changed 30% B to 100% B, 50–60 min held at 100% B. Flow rate was 0.7 mL/min, and the detection wavelength was adjusted to 280 nm.

2.3. Cell culture

Human embryonic NSCs were obtained from Life Technologies as a commercially available product (N7800-200). The cells were cultured in KnockOut DMEM/F-12 media

supplemented with Neural Supplement, 2 mM GlutaMAX, 20 ng/mL recombinant Human EGF, 20 ng/mL recombinant Human bFGF (Life Technologies) according to the manufacturer's instructions. The cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. For NSCs differentiation, the cells were cultured for 4 d in Neurobasal media supplemented with B-27 supplement, 2 mM GlutaMAX without EGF and bFGF.

2.4. MTT assay

Cell viability was calculated by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). NSCs were seeded at 1×10^4 cells per well in 96-well plate a day before the treatment. The cells were treated with various concentrations of EEGR (0.1, or 0.2 mg/mL) in 100 μL cell media for 24 h. After the treatment, MTT solution (1 mg/mL in stem cell media) was added to each well. The plates were then incubated at room temperature for 2 h, and reduced purple-blue MTT formazan crystals were solubilized by adding 100 μL of DMSO to each well. The absorbance was measured at 540 nm using a microplate ELISA reader.

2.5. Real-time RT-PCR

Total RNA was extracted from NSCs using a Trizol RNA extraction kit (Promega). Complimentary DNA was synthesized from 2 μg of total RNA using the SuperScript III system with an oligo-dT primer (Invitrogen). The primers were as follows: for Nestin, forward 5'-CAG CGT TGG AAC AGA GGT TGG-3', reverse 5'-TGG CAC AGG TGT CTC AAG GGT AG-3'; for MAP2, forward 5'-AAT GGG ATC AAC GGA GAG CT-3', reverse 5'-TCT TCA GCT GCT AAA GGC AG-3'; for Tuj1, forward 5'-AAC AGC ACG GCC ATC CAG GA-3', reverse 5'-CTT GGG GCC CTG GGC CTC CGA-3'; for Sox2, forward 5'-ATG CAC CGC TAC GAC GTG A-3', reverse 5'-CTT TTG CAC CCC TCC CAT TT-3'; for GAPDH, forward 5'-TGC ACC ACC AAC TGC TTA GC-3', reverse 5'-GGC ATG GAC TGT GGT CAT GAG-3'. All primers were purchased from Macrogen. The real-time PCRs were performed using Power SYBR Green PCR Master Mix (Applied Biosystems) with StepOnePlus Real-Time PCR system (Applied Biosystems) according to the manufacturer's instructions. All reactions were performed in triplicate using GAPDH as an internal control.

2.6. Dendritic number counting

NSCs were treated with 0.2 mg/mL of EEGR, or DMSO for control and incubated for 4 d in differentiation media. The images were randomly taken in 3 different places of plates using optical microscope (Olympus). The number of dendrites was counted in the images and divided by the numbers of cell bodies.

2.7. Double-labeled immunocytochemistry

NSCs seeded on cover-glass bottom dish were treated with 0.2 mg/mL of EEGR, or DMSO for control and incubated for 2 d in differentiation media. After the treatment, the cells were fixed in 4% paraformaldehyde in PBS pH 7.4 for 15 min at room temperature and washed in PBS. The cells were then incubated in PBS containing 0.25% Triton X-100 (PBST) at room

temperature for 10 min. After blocking with 1% BSA in PBST, the cells were incubated first with a mouse Nestin monoclonal antibody and a rabbit MAP2 antibody (Abcam) for overnight at 4 °C, and second with goat anti-mouse antibody coupled to Alexa Fluor 488 and goat anti-rabbit antibody coupled to Alexa Fluor 568 (Life Technologies) for 1 h at room temperature. The images were taken afterwards for Nestin and MAP2 expression separately, and combined Nestin and MAP2 using confocal microscopy (Olympus).

3. Results

3.1. HPLC estimation of EEGR

4-Hydroxybenzyl alcohol is one of the major components in *Gastrodiae rhizoma* with neuroprotective effects [11,17]. Therefore, we estimated whether EEGR contained 4-hydroxybenzyl alcohol or not. In Figure 1A, the peak of 4-hydroxybenzyl alcohol appeared clearly in its HPLC chromatogram.

3.2. Effect of EEGR on proliferation of embryonic NSCs under growth media

We first assessed whether EEGR altered NSC viability. MTT assay was performed using NSCs treated with 0, 0.1, 0.2 mg/mL of EEGR for 24 h. As shown in Figure 2, EEGR significantly increased NSC viability in a dose-dependent manner. Compared with control, 0.2 mg/mL of EEGR increased cell viability by

more than 20% point. These data suggest that EEGR increases NSC proliferation.

3.3. Effects of EEGR on dendrite extension of NSCs

To elucidate whether EEGR affects NSC differentiation, NSCs were treated with differentiation media with DMSO or 0.2 mg/mL EEGR for 4 d. The images taken by optical microscopy clearly showed that EEGR-treated NSCs had more extended dendritic spines, a small membranous protrusion from dendrite, than DMSO-treated NSCs did (Figure 3A). Next, we

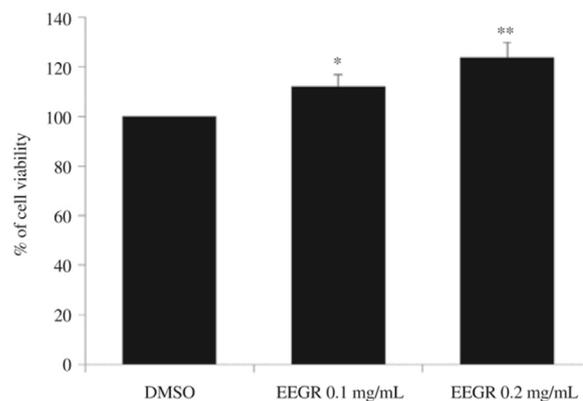
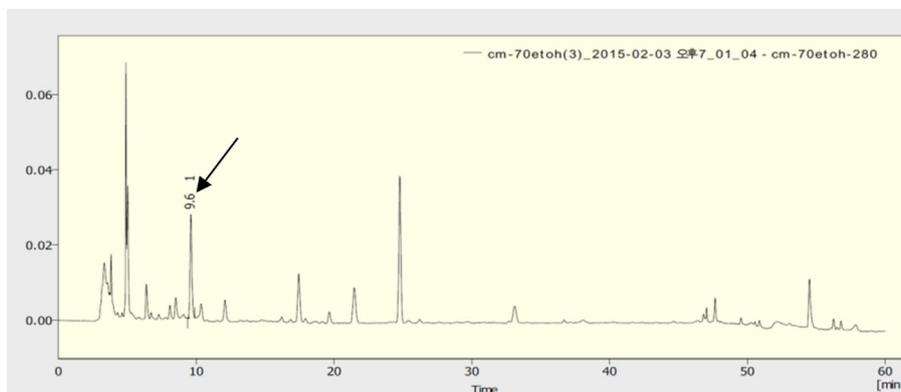
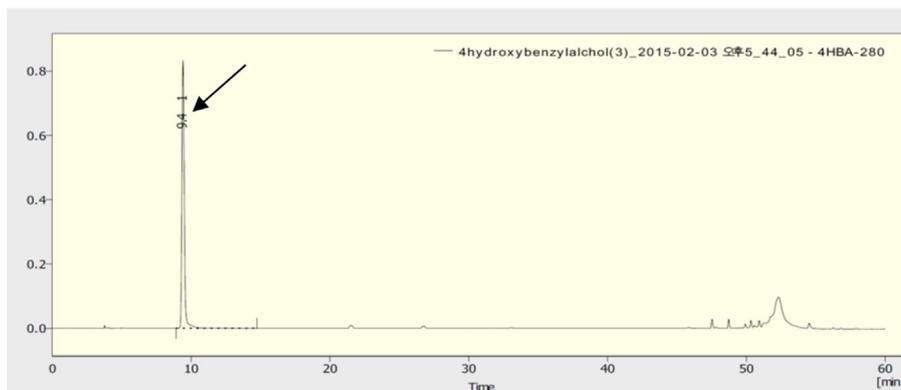


Figure 2. Effect of EEGR on NSC viability in growth media. Each bar represents the mean percentage increase above control (DMSO) ($n = 3$). Differences were statistically significant at $*P < 0.05$ and $**P < 0.01$.



(A)



(B)

Figure 1. HPLC chromatogram of EEGR. HPLC chromatogram of EEGR (A) and 4-hydroxybenzyl alcohol (B). Absorbance was measured at 280 nm. The arrow marks indicate the location of 4-hydroxybenzyl alcohol peak.

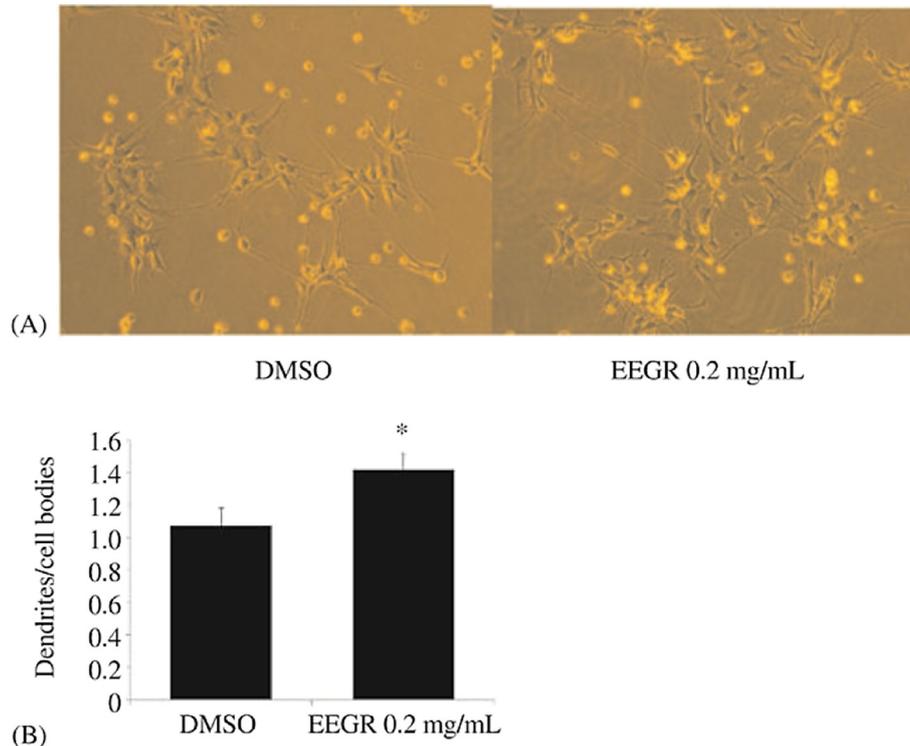


Figure 3. Effect of EEGR induces dendrite formation.

(A) The images of NSCs under optical microscopy; (B) Quantitative analysis of the number of dendrites. The number of dendrites was counted in the images and divided by the numbers of cell bodies. The bar graph shows the mean dendrite number per a NSC ($n = 3$). Differences were statistically significant at $*P < 0.05$.

counted the dendrite number per a cell body. An EEGR-treated NSC extended 1.42 dendrites per a cell, whereas a DMSO-treated NSC had 1.07 dendrites per a cell (Figure 3B). These data suggest that EEGR promotes the formation of dendrites and dendritic spines, possibly neuronal differentiation.

3.4. Effects of EEGR on neuronal differentiation of NSCs

In order to evaluate whether EEGR promotes neuronal differentiation of NSCs in a molecular level, we tested mRNA expression of marker genes such as *Nestin*, *Tuj1* and *MAP2* in differentiation process. As shown in Figure 4, EEGR decreased mRNA expression of *Nestin*, a specific marker for NSC, while it increased that of *Tuj1* and *MAP2*, neuronal differentiation markers. In accordance with optical microscopy experiment, EEGR induced 7.1-fold increase in *MAP2* expression, which is correlated with the branching ability of the neurons.

Next, we further examined double-labeled immunocytochemistry with Nestin and MAP2 antibodies (Figure 5). The expression of Nestin was shown as a button-like cluster in the cytoplasm and that of MAP2 was found associated with dendritic microtubules. MAP2 expression in EEGR-treated NSCs was localized in dendritic spines with higher area density and optical density (Figure 5B), compared to that in DMSO-treated NSCs (Figure 5A).

Because EEGR has the potent ability to differentiate NSCs, we finally examined whether EEGR induced alteration of specific gene expression even in growth media. After NSCs were cultured in growth media treated with 0, 0.1, 0.2 mg/mL of EEGR for 72 h, real-time RT-PCR analysis was conducted. Although we failed to find significant alterations in *MAP2* mRNA expression under growth media (Data not shown), EEGR decreased the mRNA expression of *SOX2*, a transcription factor essential for maintaining self-renewal, or pluripotency (Figure 6), suggesting reduction in the feature of undifferentiated stem cell.

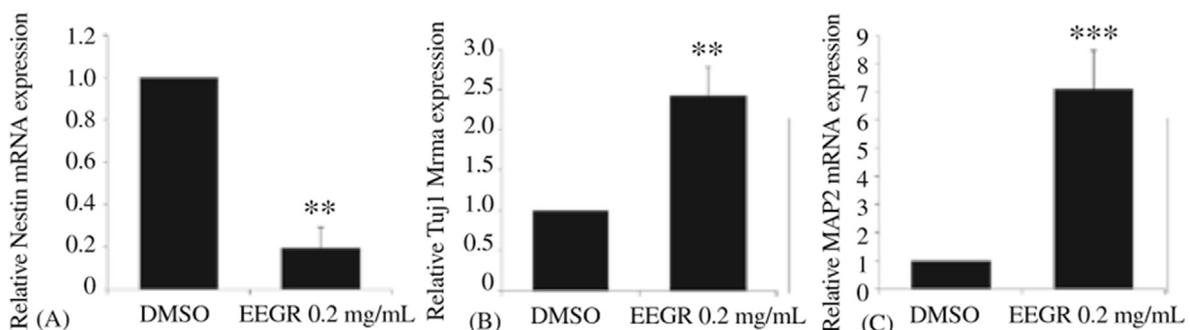


Figure 4. Effect of EEGR on mRNA expression of *Nestin*, *Tuj1* and *MAP2* in differentiation media.

(A): mRNA expression of *Nestin*, (B): mRNA expression of *Tuj1*; (C): mRNA expression of *MAP2*. Each bar represents the mean fold increase or decrease with respect to control ($n = 3$). Differences were statistically significant at $**P < 0.01$ and $***P < 0.005$.

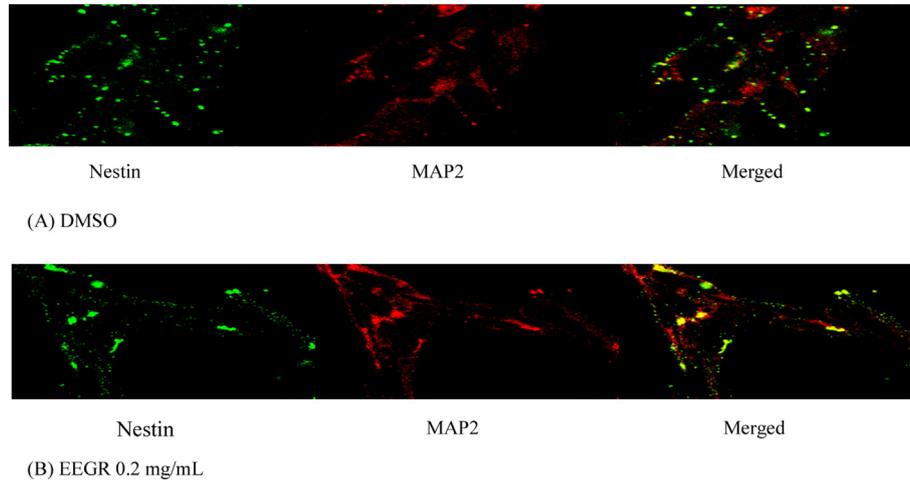


Figure 5. Effect of EEGR on MAP2 expression in dendrites.

(A): NSCs treated with DMSO; (B): NSCs treated with 0.2 mg/mL of EEGR. Double-labeled immunocytochemistry was performed using antibodies against Nestin (green) and MAP2 (red). The images are representative of at least three experiments.

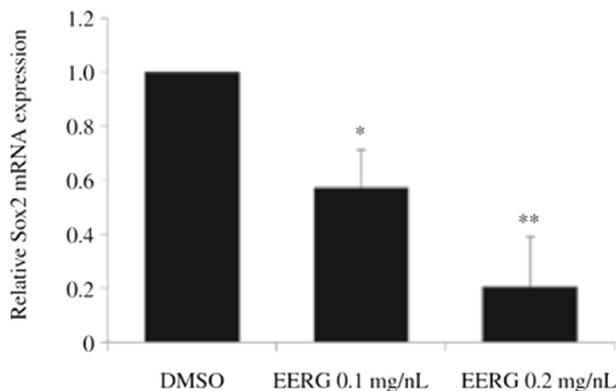


Figure 6. Effect of EEGR on mRNA expression of *Sox2* in growth media. The mRNA expression of *Sox2* was detected by real-time RT-PCR and normalized to that of GAPDH. Each bar represents the mean fold decrease below control ($n = 3$). Differences were statistically significant at * $P < 0.05$ and ** $P < 0.01$.

4. Discussion

NSCs, with their self-renewal and multiple differentiation ability, are a promising resource in cell therapies for various neurodegenerative diseases and neural tissue injuries. Although NSC properties have been extensively researched for clinical applications over the last decade [2,21], the unsolved problem is how to promote NSC proliferation effectively and induce complete differentiation into neurons, which is critical for an accurate and safe cell therapy. In this study, we demonstrate that EEGR promotes NSC proliferation and neuronal differentiation, in particular, dendrite formation; EEGR increased NSC viability under growth condition. In addition, EEGR decreased mRNA expression of *Nestin* and increased that of *Tuj1* and *MAP2*, along with increased number of dendrites and dendritic spines in differentiation process.

Similarly, *Panax notoginseng* saponins was reported to promote the proliferation and differentiation of NSCs isolated from rat hippocampus [22]. *Panax notoginseng* saponins increased the mRNA expressions of *Nestin*, *vimentin*, and *Tuj1*, suggesting differentiation of NSCs into both neurons and glial cells. In our study, EEGR decreased mRNA expression of *Nestin* and had no mRNA expression of *GFAP* detected (Data not

shown), while it increased that of *Tuj1* and *MAP2*. These results suggest that EEGR promotes NSCs differentiation into neurons, not into glial cells.

One of the most important finding in this study is that EEGR increases MAP2 expression, along with the protrusion of dendrites and dendritic spines. MAP2 has been shown to be critical for dendritic differentiation. The suppression of MAP2 using antisense oligonucleotides inhibits the outgrowth of dendrites in primary neuronal cultures [23] and MAP2 overexpression promotes the formation of cytoplasmic processes similar to dendrites in non-neuronal cells [24,25]. Therefore, it is very likely that the increased number of dendrites and dendritic spines in EEGR-treated NSCs is attributable to EEGR-induced MAP2 expression. In addition, EEGR decreased mRNA expression of *Sox2* and *Nestin*, suggesting that the properties of stem cells, self-renewal and pluripotency, disappeared in EEGR-treated NSCs. In particular, *Sox2* is a transcription factor which plays an important role in maintaining the properties of NSCs [26]. The expression of *Sox2* is localized to undifferentiated precursors, and it is generally decreased with differentiation [27]. Taken together, EEGR-mediated regulation of these gene expressions led to the differentiation of NSCs into neuronal cells, implicating its beneficial effects in NSC-based therapies.

Because *Gastrodiae rhizoma* has been widely used as a safe oriental herbal medicine for the treatment of various neurological disorders [8], the effects of its oral administration on adult NSCs should be considered. *G. elata* and its active constituents have been reported to exert neuroprotective effects [11–14], antidepressant effects [15,16], and memory improvement [17,18]. Several studies showing their beneficial effects revealed that the extracts of *G. elata* [13,19,28] or their constituents such as 4-hydroxybenzyl alcohol [11] increased neuronal cells of hippocampal CA1 region in neuronal damage or global ischemia, compared to the control. Because brain damages such as ischemic insults trigger neurogenesis from NSCs and migrate to the damaged CA1 [23], these effects on hippocampal CA1 region might be due to not only the protection from cell death, but also neurogenesis from NSCs. In a good agreement with these data, the administration of gastrodin upregulated BrdU-positive proliferation of hippocampal NSCs in depression rat model [20]. *Panax notoginseng* saponins, reported activator on NSCs proliferation and differentiation *in vitro*,

induced proliferation, migration and differentiation of adult NSCs in subventricle ventricle zone and subgranular zone of rat hippocampus after ischemia *in vivo* [29,30]. In this regard, EEGR administration also might regulate the fate of adult NSCs in the brain. Because EEGR contains various active constituents, it is likely that EEGR exhibits additive or synergistic effects of its constituents on both NSC proliferation and differentiation. To clarify this issue, further *in vivo* study will be needed.

In conclusion, EEGR increased NSC viability under growth condition, decreased expression of Nestin, and increased that of Tuj1 and MAP2, thereby, increasing the number of dendrites and dendritic spines of NSC in the differentiation process. Our data demonstrate, for the first time, that EEGR may regulate NSC proliferation and differentiation into neuronal cells. On the basis of our results, EEGR is likely to be registered as a NSC regulator in cell-based therapies for neuronal regeneration.

Conflict of interest statement

We declare that we have no conflict of interest.

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