Ginsenoside metabolite 20(S)-protopanaxadiol promotes neural stem cell transition from a state of proliferation to differentiation by inducing autophagy and cell cycle arrest

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Abstract. 20(S)-Protopanaxadiol (PPD) is an active ginseng metabolite and is the final form of protopanaxadiol saponins metabolized by human intestinal microflora. The neuroprotective effects and mechanisms underlying PPD on neural stem cells (NSCs) are not completely understood. The aim of the present study was to assess the effects of PPD on the proliferation and differentiation of neural stem cells. In the present study, following treatment with different concentrations of PPD for 24 h, the percentage of BrdU-positive cells decreased significantly with increasing concentrations of PPD. Moreover, flow cytometric analysis results indicated that PPD treatment increased the proportion of cells in the G0/G1 and G2/M phase and decreased the proportion of cells in the S phase. The activation of autophagy, determined by an increased number of autophagic vacuoles and light chain 3 lipidation, was associated with an increase in the expression of the neuronal marker tubulin-β3 following PPD treatment. PPD also partially rescued NSCs from the inhibitory effects of the autophagic inhibitor wortmannin, suggesting that the effect of PPD on NSC differentiation was associated with autophagy. Collectively, the results indicated that PPD promoted the transition of NSCs from a state of proliferation to differentiation through the induction of autophagy and cell cycle arrest. Therefore, the present study may provide a basis for the development of regenerative therapies based on ginsenoside, an approved and safe drug.

Introduction

Neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis, are a major cause of disability and death in elderly individuals, which seriously impact the physical and mental health of the affected individual (1-4). With an increasing aged population and the pressure of life, neurological diseases pose a large burden on an individual's life and health; therefore, identifying drugs that protect against neurodegeneration is important.

Ginsenosides are the active ingredients of ginseng, which has been used as a tonic drug in East Asia for >2,000 years (5). In recent years, a number of studies have reported that ginsenosides have certain roles in the prevention and treatment of neurological diseases. For example, Ginsenoside Rb1 promoted the proliferation and differentiation of neural stem cells (NSCs) in a rat model of Alzheimer's disease (6). Gerbil models of global ischemia have also indicated the significance of the ginsenosides in neurodegenerative diseases (7). However, research is still in its infancy, and effective physiological dosages and the mechanisms underlying the action of ginsenosides have not yet been fully elucidated. Ginsenosides can be classified into two categories, which display distinct pharmacological roles, based on the functional groups attached at the C6 position: 20(S)-protopanaxadiol (PPD) type and 20(S)-protopanaxatriol type (PPT) (8). In the present study, the effect of the ginsenoside de-glycosylated metabolite PPD on neurogenesis in rat NSC cultures was investigated. The results suggested that the treatment of NSCs with PPD significantly promoted the differentiation of NSCs by inducing autophagy and cell cycle arrest.
Materials and methods

**NSC cultures.** NSCs were prepared as previously described (9). A total of 160 pups were used in the present study. Postnatal day 1 Sprague-Dawley rats (specific pathogen-free grade) were purchased from the Slack Jinda Laboratory Animal Company (Hunan, China). The animals were maintained at 19-22°C with 40-50% humidity, 12-h light/dark cycles. Primary NSCs derived from postnatal day 1 rats were isolated and cultured as follows. Following sacrifice by decapitation, rat brains were dissected in ice-cold DMEM/F12 medium (1:1; Thermo Fisher Scientific, Inc.) supplemented with 2% penicillin and 50 µg/ml gentamycin. The subventricular zone was treated with 1 ml Stem Pro Accutase (Thermo Fisher Scientific, Inc.) for 10 min at 37°C. Following digestion, the samples were filtered using a 200-mesh sieve and centrifuged at 240 x g for 5 min at room temperature. The obtained NSCs (5-7x10^5) were cultured in fresh DMEM/F12 supplemented with basic fibroblast growth factor (20 ng/ml; PeproTech, Inc.), epidermal growth factor (20 ng/ml; PeproTech, Inc.), 2% B27 (Thermo Fisher Scientific, Inc.), glutamine (2 mM; Thermo Fisher Scientific, Inc.), penicillin (50 U/ml) and gentamycin (50 µg/ml; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂. The culture medium was replaced every 2 days and cells were subcultured every 5 days. Neurospheres were observed on days 1, 3 and 6 using an inverted microscope (magnification, x400; Olympus Corporation). All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Central South University (Changsha, China). All efforts were made to minimize animal suffering and reduce the number of animals used.

**Cell viability and proliferation assays.** Cell viability was assessed using the Cell Counting Kit-8 (CCK-8; Vazyme Biotech Co., Ltd.), according to the manufacturer's protocol. Ginsenoside PPD (purity, >95%) was obtained from the Department of Chemistry, Jilin University, and dissolved in DMSO to make a 100-mg/l stock solution. Neurospheres (passage 2) were digested to form single-cell suspensions. Cell suspensions (1x10^5/well) were seeded into 96-well plates in DMEM/F12 medium containing different concentrations of PPD (5, 10, 20 or 40 µM). The control group was incubated with DMEM/F12 medium containing 0.1% DMSO. Following incubation for 72 h at 37°C, 20 µl CCK-8 reagent was added to each well and incubated for a further 4 h at 37°C. Absorbance was measured at 450 nm using a microplate reader (BioTek Instruments, Inc.). The IC₅₀ was calculated using the optical density values using SPSS software (version 18.0; IBM Corp.).

Cell proliferation was assessed by BrdU incorporation. The single-cell suspensions (1x10⁶ cells/well) were plated on a 20-mm coverslip (Fisherbrand; Thermo Fisher Scientific, Inc.) in a 6-well plate in DMEM/F12 medium containing different concentrations of PPD (5, 10, 20 or 40 µM). The control group was incubated with DMEM/F12 medium containing 0.1% DMSO. Cells were co-cultured with DMEM/F12 medium containing 0.1% DMSO were used as the control group. All cells were incubated with BrdU (15 µM/l; Sigma-Aldrich; Merck KGaA) for 24 h at 37°C with 5% CO₂ subsequently assessed using an immunofluorescence assay.

**Cell cycle assay.** The cells were analyzed by flow cytometry (FC 500; Beckman Coulter, Inc.) and analyzing software (CXP 2.1; Beckman Coulter, Inc.). Cells were seeded (1x10⁶ cells/well) into 6-well plates and incubated without or with PPD (10, 20 and 40 µM) for 24 h at 37°C with 5% CO₂. Cells were harvested and permeabilized overnight at 4°C with pre-cooled 75% ethanol. Subsequently, cells were treated with 1 mg/ml RNase A for 30 min at 37°C and stained with 50 µg/ml propidium iodide in the dark for 15 min at room temperature. The cell cycle was analyzed by flow cytometry.

**Cell differentiation assay.** Single cell suspensions were seeded (3.5x10⁵ cells/well) onto 20-mm round coverslip in 6-well plates. Cells were cultured in differentiation medium (DMEM/F12 medium without growth factors, but supplemented with 5% FBS (Gibco; Thermo Fisher Scientific, Inc.) and incubated without or with PPD (10, 20, 40 and 60 µM) for 72 h at 37°C with 5% CO₂. Differentiation was measured by immunostaining and western blotting for neuronal markers tubulin-β3.

**Detection of autophagy.** Single cell suspensions (1x10⁶) were transferred into a culture bottle and cultured in differentiation medium alone or with 20 µM PPD for 24, 48 or 72 h at 37°C with 5% CO₂. Changes in the expression of autophagy markers, including LC3 and p62, were assessed by western blot analysis. Following cells cultured for 72 h, LC3 punctae formation and autophagic vacuoles were also monitored by immunofluorescence and Transmission electron microscopy (TEM).

To further assess the role of autophagy during NSCs differentiation, NSCs were treated with the autophagic inhibitor wortmannin (WM, 30 µM, Abcam; cat. no. ab120148) alone or incubated with 20 µM PPD in the differentiation medium for 72 h at 37°C with 5% CO₂. Then, neuronal marker Microtubule-Associated Protein 2 (MAP2) expression was assessed by immunofluorescence.

**Immunofluorescence.** Following fixation with 4% formaldehyde, pre-coated chamber slides containing the growing cells were washed once with PBS, permeabilized with 0.1% Triton-100 for 5 min and blocked with 0.5% BSA for 30 min. Subsequently, cells were incubated with the following primary antibodies overnight at 4°C: Mouse monoclonal anti-BrdU (1:100; Sigma-Aldrich; Merck KGaA; cat. no. NA61), Rabbit monoclonal anti-tubulin-β3 (1:100; Abcam; cat. no. ab18207) and Rabbit polyclonal anti-LC3 (1:100; Bioworld Technology, Inc.; cat. no. AP0762). Cells were washed three times with PBS and incubated for 1 h at 25°C with the goat anti-rabbit IgG or goat anti-mouse IgG (1:300; Abcam; cat. nos. ab97050 and ab97022) secondary antibodies. Cells were washed with PBS and incubated with DAPI (1:200) for 10 min. Stained cells and LC3 punctae were observed in five randomly selected fields of view using an Eclipse confocal fluorescence microscope (Nikon Corporation) and analyzed using Image J 1.46r software (National Institutes of Health).

**Western blot analysis.** Cells were washed twice with PBS and lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1.0 mM Na3VO4, 1 mM EDTA, 1% NP-40, 0.5% sodium...
deoxycholate, 0.1% SDS, 100 µg/ml phenylmethylsulfonyl fluoride, 30 µl/ml aprotinin and 4 µg/ml leupeptin, pH 7.5). Lysates were centrifuged for 10 min at 10,000 x g for 10 min at 4˚C. The protein concentrations of the collected supernatants were then determined by bicinchoninic acid Protein Assay kit (Abcam; cat. no. ab102536). Proteins (50 µg per lane) were separated via 10‑15% SDS‑PAGE for 1.5 h at 120 V and transferred to PVDF membranes for 1.5 h at 40 V. The membranes subsequently blocked with 5% skimmed milk in PBS plus 0.1% Tween‑20 (PBST) for 60 min at room temperature. The membranes were incubated overnight at 4˚C with primary antibodies (1:1,000) against Rabbit polyclonal anti‑LC3 (Bioworld Technologies, Inc.; cat. no. AP0762)), Rabbit p62 polyclonal antibody (Bioworld Technologies, Inc.; cat. no. AP6006), Rabbit monoclonal anti-tubulin-β3 (Abcam; cat. no. ab18207) and Rabbit polyclonal β‑actin (Abcam; cat. no. AP0714). LC3‑I is ~14 kDa and lipidated LC3 (LC3‑II) is ~16 kDa. Primary antibodies were diluted in 1X PBST containing 5% BSA. Following incubation, the membranes were washed with 1X PBST and incubated for 2 h at room temperature with a goat anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (1:2,500; Abcam; cat. no. ab97051). Bands were developed with ECL reagent (Thermo Fisher Scientific, Inc.; cat. no. 32209). Protein expression was quantified using Image Lab software 4.1 (Bio‑Rad Laboratories, Inc.) with β‑actin as the loading control.

TEM. At the end of incubation, cell monolayers were washed with PBS and scraped gently with a plastic cell scraper. The harvested cells were pelleted by centrifugation at 10,000 x g for 10 min at 37˚C. Subsequently, cells were fixed with 4% glutaraldehyde for 3 h at 4˚C, post-fixed with 1% perosmic acid for 2 h at room temperature, dehydrated with acetone and embedded in an epoxy resin. Serial ultrathin sections (70 nm) were placed on 400‑mesh grids, and double‑stained with 2% uranyl acetate and 0.4% lead citrate for 15 min each at room temperature. Sections were observed using a JEM1230 TEM (JEOL, Ltd.) at a magnification of x8,000 in eight randomly selected fields of view. The percentage of autophagic vacuoles area in each per field was calculated.

Statistical analysis. Statistical analyses were performed using SPSS software (version 18.0; IBM Corp.). Data are presented
 Results

PPD inhibits NSC viability and proliferation. To investigate the effect of PPD on NSC proliferation in vitro, NSCs were treated with different concentrations of PPD for 72 h. Cell viability was evaluated using the CCK-8 assay. Cell viability displayed a dose-dependent relationship with increasing concentrations of PPD (Fig. 1A); the IC_{50} of PPD was calculated as 180.0±10.2 µM. The effect of PPD on cell proliferation was further evaluated using a BrdU incorporation assay. Following treatment with different concentrations of PPD for 24 h, the percentage of BrdU-positive cells in five randomly selected fields of view was calculated for each group. The percentage of BrdU-positive cells decreased significantly with increasing concentrations of PPD compared with the percentage in the control group (Fig. 1B and C). The results indicated that PPD inhibited NSC proliferation in vitro.

PPD induces cell cycle arrest. To further investigate the effect of PPD on NSC proliferation, PPD-induced alterations to the cell cycle were investigated (Fig. 2A). Flow cytometric analysis indicated that PPD treatment increased the proportion of cells in the G_{0}/G_{1} phase in a dose-dependent manner from 69.65% in the control group to 85.10% in the 40 µM PPD group (Fig. 2B). Furthermore, PPD treatment decreased the proportion of cells in the S phase from 26.53% in the control group to 13.07% in the 40 µM PPD group (Fig. 2C). The increased proportion of cells in the G_{2}/M phase in the 10 and 20 µM PPD groups was significantly higher compared with the control group (Fig. 2D). The results suggested that PPD inhibited NSC proliferation, which may be associated with cell cycle arrest induction.
Figure 3. PPD promotes NSC differentiation. NSCs were treated with 10, 20, 40 or 60 μM PPD for 72 h. (A) Immunofluorescence images of NSC differentiation following PPD treatment, as indicated by tubulin-β3 immunostaining; scale bar, 100 μm. (B) The proportion of tubulin-β3-positive cells. "*"P<0.05 and "**"P<0.01 vs. DMSO group. NSC, neural stem cell; PPD, 20(S)-protopanaxadiol.
PPD promotes NSC differentiation by inducing autophagy.

To investigate the effect of PPD treatment on cell differentiation, immunocytochemistry and western blot assays were performed. After a 72-h culture in differentiation medium, immunofluorescence expression of the neuronal marker tubulin-β3 was evaluated. The 20 µM PPD group displayed the most significant increase in tubulin-β3-positive cells compared with the control group (P<0.05; Fig. 3A and B). Western blotting also indicated that the expression level of tubulin-β3 was significantly increased in NSCs following treatment with 20 µM PPD, compared with the control group at each respective time point (P<0.05; Fig. 4A and B). These results indicated that PPD can effectively promote the differentiation of neural stem cells, with the highest effect at 20 µm.

Therefore, in the subsequent experiments, 20 µM was selected to study the mechanism of PPD promoting the differentiation of neural stem cells.

To further investigate the effect of PPD on differentiation, NSC autophagy was assessed. LC3 is an essential component of autophagosomes that has been widely used as a marker of autophagy (10). Alterations in the protein expression levels of LC3II were determined by western blotting. It was demonstrated that 20 µM PPD treatment for 48 h significantly increased the expression of LC3II compared with the respective control group (P<0.01; Fig. 4A and C). p62 expression, which often serves as another index of autophagy, increased quickly and significantly at 24 h after PPD treatment, which was significantly earlier than that at 48 h in the control group (P<0.01 vs. DMSO group). LC3, light chain 3; PPD, 20(S)-protopanaxadiol.

Discussion

Generation of new neurons involves proliferation of progenitors, withdrawal from the cell cycle and subsequent differentiation.
Precursor cells continue to divide before acquiring a fully differentiated state, whereas terminal differentiation usually coincides with proliferation arrest and permanent exit from the cell cycle. The proportion of neural progenitors that remain in and exit from the cell cycle determines the degree of neurogenesis (11,12). The results from the present study suggested that treatment with PPD reduced the proliferation of NSCs, arrested the cell cycle at the G0/G1 and G2/M phases, and promoted NSC differentiation into neurons in vitro. Therefore, it was hypothesized that the therapeutic effect of PPD on nervous system injuries and degenerative disease may occur by promoting NSC cell cycle arrest and triggering neuronal differentiation. The results of the present study were consistent with a previous study, which reported that oral administration of ginsenoside Rb1 significantly increased cell survival but not proliferation in the hippocampus (13). Further investigation into the molecular mechanisms linking NSC cell cycle arrest to cell differentiation is required.

There is evidence for an active role for autophagy during NSC differentiation. Vázquez et al (14) reported an increase in the expression levels of the autophagy genes Autophagy Related 7, Beclin1, activating molecule in beclin1-regulated autophagy (Ambra1) and LC3 in the mouse embryonic olfactory bulb during the initial period of neuronal differentiation, along with a parallel increase in neuronal markers. Furthermore, Fimia et al (15) revealed that Ambra1 knockout in mouse embryos leads to severe neural tube defects associated

Figure 5. LC3 punctae formation and autophagic vacuoles. (A) Immunofluorescence images of autophagy following treatment with 20 µM PPD, as indicated by LC3 immunostaining (scale bar, 100 µm). (B) The percentage of cells with LC3 punctae. (C) Transmission electron microscopy images displayed autophagosome formation in NSCs following treatment with 20 µM PPD for 72 h, the arrows indicate autophagic vacuoles. scale bar, 2 µm. (D) The percentage of autophagic vacuoles area in each per field. *P<0.05 vs. the DMSO group. LC3, light chain 3; PD, 20(S)-protopanaxadiol.
with autophagy impairment, the accumulation of ubiquitinated proteins, unbalanced cell proliferation and excessive cell death. Chemical inhibitors, including 3-methyladenine and LY294002, can reverse retinoic acid-induced neuronal differentiation of neuroblastoma N2a cells, and RNA interference of Beclin 1 significantly delays this process (16).
Results from the present study indicated that LC3II expression was significantly increased following treatment with PPD for 48 h compared with the control group. The p62 expression, which often serves as another index of autophagy, increased quickly and significantly at 24 h after PPD treatment, which was significantly earlier than 48 h in the control group. Previous studies have reported that p62 protein, via LC3, might be involved in facilitating the clearance of polyubiquitinated protein aggregates by linking the aggregates to the autophagic machinery (17,18). Deterioration of the p62 promoter results in a blockade of NSC proliferation and promoting cell differentiation. Future studies investigating the mechanisms underlying the effects of PPD on NSC differentiation and survival are required to verify the results of the present study.

In conclusion, the results indicated that PPD inhibited NSC proliferation and promoted NSC differentiation, potentially through a mechanism associated with autophagy and cell cycle arrest. However, the present study was only preliminary and included a number of limitations, such as the lack of in vivo experiments and a failure to present data regarding alterations to the expression levels of LC3II and tubulin-β3 in the presence of the autophagy inhibitor WM. The present study may provide a theoretical basis for the development of novel regenerative therapeutic strategies using ginsenoside, an approved and safe drug.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors' contributions
ZL, QW and JL conceived and designed the study, SC, JH, XQ, TL, SL and AP performed the experiments and data analyses. ZL, QW, SC and AP drafted the manuscript and figures.

Ethics approval and consent to participate
The present study was approved by the Institutional Animal Care and Use Committee of Central South University.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References
1. Blin P, Dureau-Pournin C, Foubert-Samier A, Grolleau A, Corbillion E, Jové J, Lassalle R, Robinson P, Poutignat N, Droz-Perroteau C and Moore N: Parkinson's disease incidence and prevalence assessment in France using the national health-care insurance database. Eur J Neurol 22: 464-471, 2015.
2. Bhuirkr KS and Rupasinghe HP: Polyphenols: Multipotent therapeutic agents in neurodegenerative diseases. OxiMed Cell Longev 2013: 891748, 2013.
3. Rocca WA, Petersen RC, Knopman DS, Hebert LE, Evans DA, Hall KS, Gao S, Unverzagt FW, Langa KM, Larson EB and White LR: Trends in the incidence and prevalence of Alzheimer's disease, dementia, and cognitive impairment in the United States. Alzheimers Dement 7: 80-93, 2011.
4. Davinelli S, Maes M, Corbi G, Zarrelli A, Willcox DC and Scapagnini G: Dietary phytochemicals and neuro-inflammaging: From mechanistic insights to translational challenges. Immun Ageing 13: 16, 2016.
5. Shin BK, Kwon SW and Park JH: Chemical diversity of ginseng saponins from Panax ginseng. J Ginseng Res 39: 287-298, 2015.
6. Zhao L, Lu S, Yu H, Duan S and Zhao J: Autophagy promotes the proliferation and differentiation of neural stem cells in Alzheimer's disease model rats. Brain Res 1678: 187-194, 2015.
7. Cheng Y, Shen LH and Zhang JT: Anti-amnestic and anti-ageing effects of ginsenoside Rg1 and Rb1 and its mechanism of action. Acta Pharmacol Sin 26: 143-149, 2005.
8. Wong A, Che C and Leung K: Recent advances in ginseng as cancer therapeutics: A functional and mechanistic overview. Nat Prod Rep 32: 256-272, 2015.
9. Marshall GP II, Ross HH, Suslow O, Zheng T, Steindler DA and Laywell ED: Production of neurospheres from CNS tissue. Methods Mol Biol 438: 135-150, 2008.
10. Kliomsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, Adachi H, Adams CM, Adams PD, Adeli K, et al: Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Autophagy 12: 1-222, 2016.
11. Ruijtenberg S and van den Heuvel S: Coordinating cell proliferation and differentiation: Antagonism between cell cycle regulators and cell type-specific gene expression. Cell Cycle 15: 196-212, 2016.
12. Zhang RL, Zhang ZG, Roberts C, LeTourneau Y, Lu M, Zhang L, Wang Y and Chopp M: Lengthening the G(1) phase of neural progenitor cells is concurrent with an increase of symmetric neurosphere generating division after stroke. J Cereb Blood Flow Metab 28: 602-611, 2008.
13. Liu L, Hoang-Gia T, Wu H, Lee MR, Gu L, Wang C, Yun BS, Wang Q, Ye S and Sung CK: Ginsenoside Rb1 improves spatial learning and memory by regulation of cell genesis in the hippocampal subregions of rats. Brain Res 1382: 147-154, 2011.
14. Vázquez P, Arroba AI, Cecconi F, de la Rosa EJ, Boya P and de Pablo F: Atg5 and Ambral differentially modulate neurogenesis in neural stem cells. Autophagy 8: 187-199, 2012.
15. Finnia GM, Stoikova A, Romagnoli A, Giunta L, Di Bartolomeo S, Nardiacci R, Corazzari M, Fucco C, Ucar A, Schwartz P, et al: Ambral regulates autophagy and development of the nervous system. Nature 447: 1121-1125, 2007.
16. Zeng M and Zhou JN: Roles of autophagy and miTOR signaling in neuronal differentiation of mouse neuroblastoma cells. Cell Signal 20: 659-665, 2008.
17. Shivets E, Fass E, Scherz-Shouval R and Elazar Z: The N-terminus and Phe52 residue of LC3 recruit p62/SQSTM1 into autophagosomes. J Cell Sci 121: 2685-2695, 2008.
18. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, Øvervatn Ø, Bjørkøy G and Johansen T: P62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem 282: 24131-24145, 2007.