Icariin promotes cell proliferation and regulates gene expression in human neural stem cells *in vitro*

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Abstract. Icariin (ICA), which is an essential bioactive component extracted from the herb *Epimedium*, possesses neuroprotective properties. The aim of the present study was to investigate the regulatory roles of ICA in cell proliferation and gene expression in human neural stem cells (NSCs) *in vitro*. Single cells were isolated from the corpus striatum of 16-20-week human fetuses obtained following spontaneous abortion. The cells were cultured in Dulbecco's modified Eagle's medium/F12 complete medium and were characterized by immunostaining and cell differentiation assay. NSCs were treated with ICA, and cell proliferation was assessed using the Cell Counting kit-8 cell proliferation assay kit. In addition, neurosphere formation was comparatively studied between the ICA-treated and control cells. cDNA microarray analysis was performed to examine the effects of ICA on gene expression. Altered expression of genes important for regulating NSC proliferation was further analyzed by quantitative polymerase chain reaction (qPCR). The results demonstrated that typical neurospheres appeared after 7-10 days of culturing of individual cells isolated from the corpus striatum. These cells expressed nestin, an important NSC marker, and in the presence of differentiation medium they expressed β-III-tubulin, a specific neuronal marker, and glial fibrillary acidic protein, an astrocyte marker. Treatment with ICA enhanced NSC proliferation and the formation of neurospheres. Microarray data and pathway analysis revealed that the genes regulated by ICA were involved in several signaling pathways, including the Wnt and basic fibroblast growth factor (bFGF) pathways, which are important for the regulation of NSC function. Upregulation of frizzled class receptor 7 and dishevelled segment polarity protein 3, which are key players in the Wnt pathway, and fibroblast growth factor receptor 1, which is the receptor for bFGF, and downregulation of glycogen synthase kinase-3β, which is a Wnt pathway inhibitor, was further validated by qPCR. In conclusion, ICA promoted proliferation and regulated gene expression in human NSCs, thus suggesting that ICA may exert its neuroprotective effects by regulating NSC activity.

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Introduction

Neural stem cells (NSCs) are multipotent stem cells, which are derived from neural tissues, either from the central or peripheral nervous systems. NSCs are self-renewing and can give rise to various cell types of the nervous system, including neurons, astrocytes and oligodendrocytes, through asymmetric cell division (1). NSC-derived neurogenesis is critical for various aspects of central nervous function, including spatial learning and memory, mood regulation and motor control (2-4). Previous studies have suggested that NSC transplantation may have beneficial effects in the treatment of numerous pathological conditions, such as neurodegenerative diseases, seizures and brain tumors (5-7). Therefore, NSCs may be considered a promising therapeutic option for the treatment of ischemic stroke and neurodegenerative diseases (8). Nevertheless, how to promote cell survival and proliferation following cell transplantation remains a significant challenge for stem cell therapy using NSCs to treat various neurodegenerative disorders and strokes.

Epimedium is a plant of the *Epimedium brevicornum* Maxim species of the Berberidaceae family, which contains various bioactive compounds, including flavonoids and alkaloids (9). Icariin (ICA) is a type of flavonoid, and is the most metabolically active component extracted from *Epimedium*. ICA possesses various bioactivities, including antidepressant-like effects and anti-inflammatory activity (10,11). In addition, it has previously been reported that ICA improves memory impairment in a murine model of Alzheimer's disease, and ameliorates motor dysfunction in spinal cord injury in mice (12,13). Due to the known actions of ICA, and the important role NSCs have in neuroregeneration, the present

study hypothesized that ICA may stimulate cell proliferation and regulate gene expression in NSCs.

Materials and methods

Materials. ICA (purity >98%, high-performance liquid chromatography grade) with the molecular formula $C_{33}H_{42}O_{15}$ and a molecular weight of 676.65 was purchased from Shaanxi Scidoor Hi-tech Biology Co., Ltd. (Xi'an, China). Hank's Balanced Salt Solution (HBSS), Dulbecco's modified Eagle's medium (DMEM)/F12 medium, fetal bovine serum (FBS), B27, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), Glutmax and poly-L-lysine-coated plates were purchased from Thermo Fisher Scientific, Inc. (Beijing, China). Mouse anti-nestin was obtained from BD Pharmingen (cat. no. 560341; San Jose, CA, USA), mouse anti-β-III-tubulin (cat. no. T8578), mouse anti-glial fibrillary acidic protein (GFAP; cat. no. G3893) and rabbit anti-galactocerebroside (GalC; cat. no. G9152) were obtained from Sigma Aldrich (Shanghai) Trading. Co., Ltd.(Shanghai, China). Cy2- or Cy3-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). RNeasy kit and AMV reverse transcriptase were obtained from Qiagen, Inc. (Valencia, CA, USA). Cy3-dCTP and Cy5-dCTP were purchased from CapitalBio, Inc. (Beijing, China). SYBR Premix Ex Taq for quantitative polymerase chain reaction (qPCR) was obtained from Sangon Biotech Co., Ltd. (Shanghai, China).

NSC isolation and characterization. The use of human fetuses obtained following spontaneous abortion was approved by the Ethic Committee of Xuanwu Hospital of Capital Medical University (Beijing, China), and written informed consent was obtained from the study participants. The corpus striatum of 16-20-week fetuses were obtained from 5 fetuses following spontaneous abortion, and were dissected and chopped in cooled HBSS using a tissue chopper. The tissue was further triturated by pipetting up and down 10 times to dissociate cells. After filtering through a 300 micron stainless steel sieve, in order to remove the large pieces of tissue, the dissociated cells were pelleted by centrifugation at 200 x g for 5 min at room temperature, washed once with HBSS, and were re-suspended at a density of $2x10^6$ cells/ml in DMEM/F12 medium containing 2% B27, 20 ng/ml EGF, 20 ng/ml bFGF and 1% Glutmax (DMEM/F12 complete medium). Cells were seeded in 100 mm dishes and cultured at 37˚C in a humidified atmosphere containing 5% CO_2. Cell medium was changed every 3 days. After an initial culture for 7-10 days typical NSC clusters appeared, which were pelleted by centrifugation at 200 x g for 5 min at room temperature and were treated with accutase. Accutase-digested cells were washed once with HBSS, re-suspended in DMEM/F12 complete medium and used for all subsequent assays. For NSC characterization, the cells were seeded onto poly-L-lysine-coated chamber slides ($1x10^4/cm^2$). Following a 12 h culture, the cells were subjected to immunostaining for the analysis of nestin expression.

NSC differentiation. Growth factor-free DMEM/F12 medium supplemented with 1% FBS was used to induce cell differentiation. Cell clusters were digested with accutase as aforementioned, were washed once with HBSS, re-suspended in differentiation medium, and were seeded onto poly-L-lysine-coated chamber slides ($1x10^4/cm^2$). Cells were cultured for 7 days, and were subsequently subjected to fixation for immunostaining, in order to determine the expression of β-III-tubulin, GFAP and GalC.

Immunostaining. Cells were fixed in ice-cold 4% paraformaldehyde for 15 min, followed by three washes with phosphate-buffered saline (PBS; pH 7.4). After blocking with 10% goat serum (Jackson ImmunoResearch, Inc., West Grove, PA, USA) in PBS containing 0.3% Triton X-100 at 37˚C for 1 h, the cells were incubated with various primary antibodies at 4˚C overnight. The antibodies used were as follows: Mouse anti-nestin (1:1,000), mouse anti-β-III-tubulin (1:1,000), mouse anti-GFAP (1:500) and rabbit anti-GalC (1:100). After three washes with PBS, Cy2- or Cy3-conjugated secondary antibodies (1:500) were added to the cells and were incubated at 37˚C for 30 min, followed by three washes with PBS. Cells were then mounted with VectaShield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA) and 4',6-diamidino-2-phenylindole was used for nuclear counterstaining. Fluorescent signals were visualized using a confocal laser microscope system (MRC1024; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell treatment and proliferation assay. To study the effects of ICA on NSC proliferation, accutase-dissociated single cells were suspended in DMEM/F12 complete medium at a density of $2x10^5$ cells/ml. A 100 μl cell suspension was added into each well of a 96-well plate, which had previously been coated with poly-L-lysine. Following a 24 h culture, the medium was replaced with serum- and growth factor-free medium containing various concentrations of ICA (final concentrations, 0.1, 1 and 10 μM). After a further 24 h of culture, the medium was removed and serum- and growth factor-free medium (without phenol red) containing 10% Cell Counting kit (CCK)-8 reagent was added (Dojundo Molecular Technologies, Inc., Kumamoto, Japan). The cells were cultured for 4 h at 37˚C, and the optical density (OD) of each well was measured at 450 nm (OD450) using a microplate reader. Cells cultured in serum- and growth factor-free medium served as a control. In a separate experiment, individual NSCs in DMEM/F12 complete medium were seeded into each well of a 24-well plate (10^4 cells/cm^2) coated with poly-L-lysine. Following a 24 h culture, the medium was replaced with serum- and growth factor-free medium containing 10 μM ICA (final concentration). Cells were maintained in culture for 7 days, and the formation of neurospheres was observed by microscopy. The diameters of 15 randomly chosen neurospheres from each well were measured using a micrometer installed in the microscope (IX70; Olympus Corporation, Tokyo, Japan). The results from three wells of each treatment group were averaged and compared.

cDNA microarray analysis. cDNA microarray analysis was performed for gene expression profiling, and the results were compared between the cells treated with ICA and the untreated control cells. Cell treatment with 10 μM ICA was conducted, as aforementioned. NSCs cultured in serum- and growth factor-free medium without ICA served as a control. RNA was extracted from the cells using the RNeasy kit, according

Table I. Quantitative polymerase chain reaction primer sequences.

Genes	Reference number	Sequence	Product size
FZD7	NM_003507	F: 5'-GGCGCCTCTGTTCGTCTAC-3' R: 5'-GGTCTTGGTGCCGTCGTGT-3'	106 bp
CTNNB1	NM_001904	F: 5'-CACGTGCAATCCCTGAACTGA-3' R: 5'-CGCATGATAGCGTGTCTGGAA-3'	121 bp
DVL3	NM_004423	F: 5'-GGTACTGCGGGAGATTGTG-3' R: 5'-GGAAGGTGCCGGTCAT-3'	168 bp
GSK-3β	NM_002093	F: 5'-GTCCGATTGCGTTATT-3' R: 5'-TTCGGAACAGCTGATACAT-3'	176 bp
FGFR1	NM_023105	F: 5'-AAGGCATCATTTGGTGAACAGAAC-3' R: 5'-TCACACAACATTGCTTCAAGGTAGG-3'	133 bp
GAPDH	NM_002046	F: 5'-ATGACATCAAGAAGGTGGTG-3' R: 5'-CATACCAGGAAATGAGCTTG-3'	177 bp

F, forward primer; R, reverse primer; bp, base pair; FZD7, frizzled class receptor 7; CTNNB1, catenin beta-1; DVL3, dishevelled segment polarity protein 3; GSK-3β, glycogen synthase kinase-3β; FGRF1, fibroblast growth factor receptor 1; GAPDH, glyceraldehyde 3-phosphate dehyrdrogenase.

to manufacturer's protocol, and was quantified using the spectrophotometric method. Total RNA (2 μg) was used for first-strand cDNA synthesis using AMV reverse transcriptase, according to the manufacturer's protocol. Cy3-dCTP was added to the cDNA of the control cells, whereas Cy5-dCTP was added to the cDNA of ICA-treated cells. Microarray analysis was performed using the 22 K Human Genome Array (CapitalBio, Inc.). The slide contained gene-specific 70-mer oligonucleotides representing 21,329 human genes, including four human housekeeping genes as positive controls, and 12 negative controls, which were designed to have no significant homology with known human gene sequences. The labeled samples were quantitatively adjusted based on the efficiency of Cy-dye incorporation and mixed into 80 μl hybridization solution [3X saline-sodium citrate (SSC), 0.2% sodium dodecyl sulfate (SDS), 25% formamide and 5X Denhardt's). cDNA in hybridization solution was denatured at 95˚C for 3 min prior to loading onto the microarray. The array was hybridized at 42˚C overnight, and washed once with washing solution (0.2% SDS and 2X SSC) at 42˚C for 5 min, followed by one wash with 0.2% SSC at room temperature for 5 min. Finally, the array was scanned using a confocal LuxScan 10 KA scanner (CapitalBio, Inc.). The data of the obtained images were extracted using LuxScan 3.0 software (CapitalBio, Inc.). Genes with a signal intensity >800 (Cy3 or Cy5) were considered to be expressed. In every two channel slides, the intensity ratio of Cy5 to Cy3 of each spot was calculated following normalization with LOWESS regression. Statistical data and differential analysis files were generated using SAM software 3.0 (Stanford University, Stanford, CA, USA). Significantly altered genes were selected based on a $P<0.05$ and >2 fold change. All differentially expressed genes were analyzed using the free web-based Molecular Annotation System 2.0 (MAS 2.0; http://bioinfo.capitalbio.com/mas3/).

qPCR. qPCR was performed to verify the array results. Primers (Table I) were designed based on published gene sequences using the Oligo 5.0 program (Molecular Biology Insights, Inc., Colorado Springs, CO, USA), and were synthesized by Sangon Biotech Co., Ltd. The PCR mix (20 μl) contained 10 μl 2X SYBR Taq Premix buffer, 1 μl cDNA, 200 nM of each primer (final concentration) and 9.0 μl nuclease-free water. The reaction mixture was incubated at 95˚C for 10 sec, followed by 40 amplification cycles at 95˚C for 5 sec, 60˚C for 10 sec and 72˚C for 10 sec. A melting curve analysis was run to determine the specificity of amplification following each qPCR analysis. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (14), following normalization against glyceraldehyde 3-phosphate dehyrdrogenase levels.

Statistical analysis. All results are expressed as the mean ± standard error of the mean, and were analyzed using one-way analysis of variance followed by Tukey's post-hoc test using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

NSC isolation and characterization. NSCs were isolated from the corpus striatum of 16-20-week human fetuses obtained following spontaneous abortion. After culturing in DMEM/F12 complete medium for 7-10 days, typical NSC clusters appeared in the culture. Immunostaining analysis revealed that neurosphere-forming cells expressed nestin, a marker of NSCs (Fig. 1A). Cell differentiation assay demonstrated that NSCs were able to differentiate into different cell types of the nervous system, as indicated by the expression of β-III-tubulin, a specific neuronal marker (Fig. 1B); and GFAP, an astrocyte marker (Fig. 1C). Few cells expressed GalC, an oligodendrocyte marker (data not shown).

NSC proliferation is stimulated by icariin in vitro. Using a CCK-8 cell proliferation assay kit, the effects of ICA on NSC proliferation were determined. As shown in Fig. 2, NSCs were

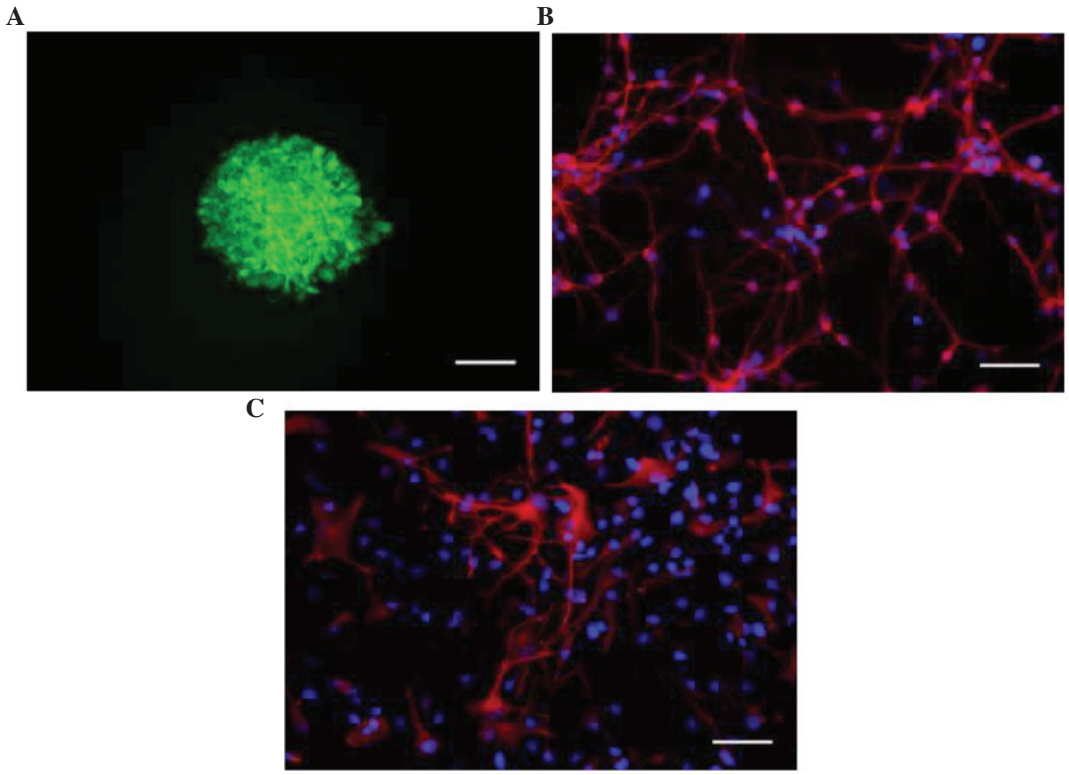

Figure 1. Isolation and characterization of neural stem cells (NSCs). Cells isolated from the corpus striatum were cultured in Dulbecco's modified Eagle's medium/F12 complete medium and were characterized. (A) Representative image of nestin-positive cells (green) in a neurosphere. As shown by immunostaining, when NSCs were cultured in differentiation medium they expressed (B) β-III-tubulin, a neuronal marker [red, β-III-tubulin-positive; blue, 4',6-diamidino-2-phenylindole (DAPI) nuclear staining], and (C) glial fibrillary acidic protein (GFAP), an astrocyte marker (red, GFAP-positive; blue: DAPI nuclear staining). Scale bar, 100 μm.

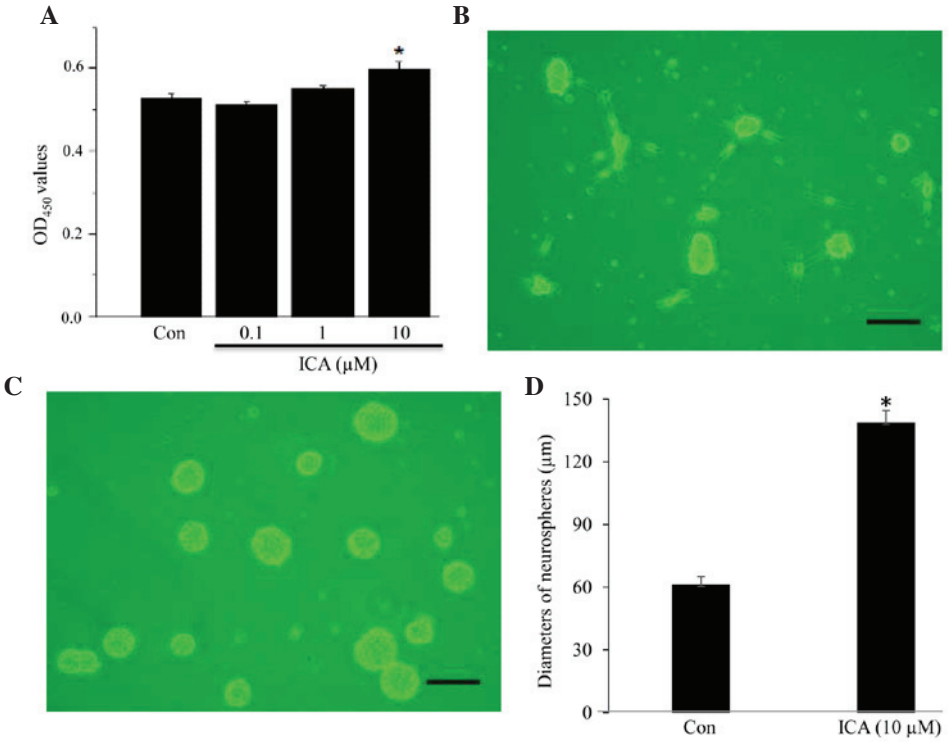

Figure 2. Icariin (ICA) promoted neural stem cell (NSC) proliferation. NSCs were treated with various concentrations of ICA, as indicated, and cell proliferation was assessed using the Cell Counting kit-8 cell proliferation assay kit. (A) Cells incubated with 10 μM ICA exhibited a significantly higher proliferation rate compared with the control cells. When cells were treated with 50 μM ICA, apparent cell toxicity was observed (data not shown). In a separate experiment, the effects of ICA on neurosphere formation were examined. Representative images of neurospheres formed in the (B) control and (C) ICA-treated groups. (D) The diameter of neurospheres formed in the ICA-treated group was significantly greater than that of neurospheres in the control group. Data are presented as the mean ± standard error of the mean. *P<0.05, compared with control cells. Scale bar, 200 μm. Con, control cells; OD, optical density.

Table II. Differences in gene expression in the top 10 pathways analyzed using Molecular Annotation System software.

Pathway	Total genes	P-value	Genes
Regulation of actin cytoskeleton	19	2.0E-8	ITGA7↑, PDGFRA↑, ACTN1↑, PFN1↑, GSN↑, PDGFRB↓, GNG12↑, FGFR2↑, FGD3↓, PAK3↓, APC2↓, FGFR1↑, PFN2↓, ARHGEF7↓, ITGA2↑, ITGB5↑, TIAM1↓, PIP5K1B↓, FGFR3↓
Focal adhesion	22	2.3E-7	CAST↑, ITGA7↑, PDGFRA↑, JUN↑, ACTN1↑, LAMC1↑, PDGFRB↓, COL1A2↑, TNC↑, CCND1↑, SHC3↑, THBS4↑, THBS2↓, MAPK10↓, PAK3↓, SPP1↑, ITGA2↑, COL4A1↑, ITGB5↑, PDGFD↓, LAMA4↑, SRC↑
MAPK pathway	20	5.2E-7	PRKX↓, MEF2C↓, PDGFRA↑, JUN↑, CACNB3↓, DUSP4↑, NTRK2↓, DUSP6↑, PDGFRB↓, MYC↑, GNG12↑, FGFR2↑, MAPK10↓, CACNG4↓, MKNK1↓, GADD45A↑, FGFR1↑, TNFRSF1A↑, HSPA5↑, FGFR3↓
ECM-receptor interaction	12	8.5E-7	ITGA7↑, SDC3↑, LAMC1↑, COL1A2↑, TNC↑, THBS4↑, THBS 2↓, SPP1↑, ITGA2↑, COL4A1↑, ITGB5↑, LAMA4↑
Axon guidance	12	2.0E-6	EPHA3↓, NCK2↓, EPHA2↑, PAK3↓, SEMA6D↓, ROBO2↓, RGS3↑, DPYSL2↓, SEMA5B↓, PLXNA2↓, EPHB3↓, EPHB4↓
Cell communication	11	7.0E-6	INA↓, LAMC1↑, GJA1↓, COL1A2↑, TNC↑, THBS4↑, THBS2↓, VIM↑, SPP1↑, COL4A1↑, LAMA4↑
TGF-beta pathway	9	1.2E-5	ACVR1↓, RPS6KB2↓, MYC↑, THBS4↑, THBS2↓, AMH↓, INHBB↓, LTBP1↑, BMP7↑
Wnt pathway	10	3.8E-5	WNT7B↑, FZD7↑, DVL3↑, GSK- 3β↓, MYC↑, CCND1↑, CTNNB1↑, TCF7↑, LEF1↑, JUN↑
Gap junction	9	5.2E-5	TUBB3↓, PRKX↓, PDGFRA↑, PDGFRB↓, EDG2↑, GJA1↓, SRC↓, PDGFD↓, CSNK1E↓
Glutathione metabolism	6	5.9E-5	GSS↑, GSTA4↓, GSTO1↑, MGST2↑, GSTZ1↑, GPX7↑

↑Indicates gene upregulation and ↓indicates gene downregulation by icariin. MAPK, mitogen-activated protein kinase; ECM, extracellular matrix; TGF, transforming growth factor.

Table III. Differentially expressed genes in the Wnt signaling pathway.

Gene	Fold change (ICA/control)	Molecular functions
WNT7B	2.482	Wnt ligand
FZD7	2.850	Non-G-protein coupled receptor
DVL3	2.191	Catalytic activity
GSK-3β	0.468	Kinase and inactivating agent
MYC	4.390	Transcription regulator
CCND1	11.677	Mitotic and cell cycle regulation
CTNNB1	2.500	Coactivator of transcription
TCF7	2.804	RNA polymerase II transcription
LEF1	10.663	Transcription regulator
JUN	2.086	Transcription cofactor

ICA, icariin.

incubated with various concentrations of ICA. In the presence of 0.1 or 1 μM ICA, cells did not exhibit markedly increased proliferation compared with the control cells (Fig. 2A). Conversely, cells incubated with 10 μM ICA exhibited a significantly increased proliferation rate compared with the untreated cells (n=4, P<0.05; Fig. 2A). When ICA concentration increased to 50 μM, cell toxicity was observed (data not shown). In a separate assay, the formation of neurospheres under the influence of ICA was examined. Neurospheres formed in ICA-treated cells appeared larger compared with those in the control group (Fig. 2B and C). By measuring neurosphere diameter, it was demonstrated that the diameter of neurospheres formed in the ICA-treated group was significantly greater than that of the neurospheres in the control group (n=5, P<0.05; Fig. 2D).

Gene expression profiling by cDNA microarray. Gene expression in 10 μM ICA-treated cells and control cells was profiled by cDNA microarray, and the results were compared. A total of 779 genes were differentially expressed between the ICA-treated group and the control group, based on P<0.05 and >2 fold change criteria (data not shown). Of the altered genes, those involved in the top 10 cell signaling pathways analyzed using MAS software were listed in Table II. Since the Wnt signaling pathway and the bFGF pathway have important roles in NSC proliferation and neurogenesis, several differentially expressed genes important in the Wnt signaling pathway were selected (Table III). Fibroblast growth factor receptor 1 was also further analyzed by qPCR to validate the array results.

Gene expression validation by qPCR. The mRNA expression levels of fibroblast growth factor receptor 1 (FGFR1), and four genes of the Wnt signaling pathway: Frizzled class receptor 7 (FZD7), dishevelled segment polarity protein 3 (DVL3), catenin beta-1 (CTNNB1) and glycogen synthase kinase-3β (GSK-3β), were further determined by qPCR. The results indicated that the mRNA expression levels of FZD7 (Fig. 3A) and DVL3 (Fig. 3B) were significantly elevated in the ICA-treated group compared with the control group (P<0.01

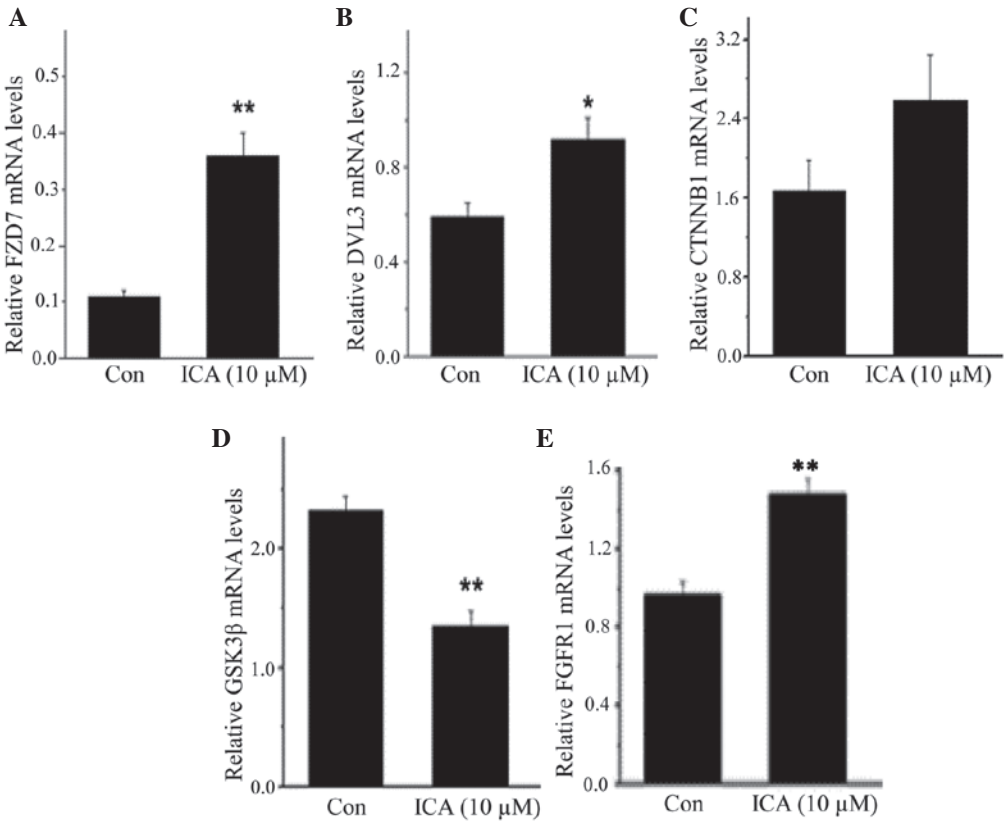

Figure 3. Icariin (ICA) regulated gene expression in neural stem cells. cDNA microarray analysis identified genes whose expression was altered following ICA treatment (Tables II and III). Using quantitative polymerase chain reaction, the mRNA expression levels of (A) frizzled class receptor 7 (FZD7), (B) dishevelled segment polarity protein 3 (DVL3), (C) catenin beta-1 (CTNNB1) and (D) glycogen synthase kinase (GSK)-3β, which are key players in the Wnt signaling pathway, and (E) fibroblast growth factor receptor 1 (FGFR1), which is the receptor for fibroblast growth factor, were determined. In the presence of ICA, the expression levels of FZD7, DVL3 and FGFR1 was significantly increased, whereas GSK-3β was markedly reduced, verifying the microarray data (n=5 for each gene). Data are presented as the mean ± standard error of the mean. *P<0.05, **P<0.01, compared with control cells. Con, control cells.

and P<0.05, respectively); CTNNB1 expression was increased in ICA-treated cells; however, the increase was not significantly different compared with the control cells, as determined by statistical analysis (Fig. 3C). The mRNA expression levels of GSK-3β were markedly reduced in the ICA-treated cells (n=6, P<0.01; Fig. 3D). In addition, FGFR1 expression was significantly upregulated by ICA (P<0.01; Fig. 3E).

Discussion

NSCs possess the characteristics of self-renewal and multipotency (1-5). The present study isolated and cultured NSCs from the corpus striatum of 16-20-week fetuses obtained following spontaneous abortion, and demonstrated that these cells expressed nestin, which is an intermediate filament protein selectively expressed in NSCs and commonly used as a marker for NSCs (15). Cultured in differentiation medium, the NSCs expressed β-III-tubulin, a specific neuronal marker, and GFAP, a marker for astrocytes (16,17), thus indicating that the NSCs were multipotent.

In our previous study, *Epimedium* flavonoids were revealed to promote the proliferation and differentiation of NSCs derived from neonatal rats *in vitro* (9). ICA, which is an important flavonoid extracted from *Epimedium*, has not yet been investigated regarding its role in regulating cell proliferation and gene expression in NSCs. The present study examined the effects of ICA on NSC proliferation. The results demonstrated that NSCs treated with 10 μM ICA exhibited a significantly higher proliferation rate compared with untreated cells. When NSCs were incubated with 10 μM ICA for 7 days, larger neurospheres were formed compared with those in the control group. Measurement of neurosphere diameter revealed the diameter of neurospheres formed in the ICA-treated group was significantly greater compared with in the control group. These data suggested that ICA promoted NSC proliferation and growth.

In order to provide information regarding the mechanisms underlying ICA actions, the present study analyzed ICA-regulated gene expression in NSCs. A cDNA microarray analysis, which is an important tool used to uncover the molecular basis of several biological processes (18), was used to profile gene expression in NSCs. The results indicated that treatment with ICA regulated gene expression in NSCs, affecting numerous signaling pathways (Tables II and III). Of these pathways, the Wnt signaling pathway has been recognized as a key regulator for self-renewal and maintenance of neural progenitors during neurogenesis (19-21), and bFGF signaling has been reported to potently induce NSC proliferation (22-25). The canonical Wnt pathway cascade is initiated when Wnt signaling proteins bind to Frizzled protein receptors, such as FZD7, which is a family of G protein-coupled receptors (26-28). Subsequently, the receptor interacts with a downstream mediator, the Dishevelled protein, which is an

essential effector for the accumulation of β-catenin encoded by CTNNB130 (27,29,30). The accumulated β-catenin then translocates to the nucleus where it regulates target gene expression to control cell function, including cell proliferation. Conversely, GSK-3β negatively regulates Wnt signaling (27,31). The microarray analysis conducted in the present study demonstrated that the expression levels of several genes in the Wnt and bFGF pathways were altered following ICA treatment. The expression levels of FGFR1, and four important Wnt family members: FZD7, DVL3, CTNNB1 and GSK-3β were further determined by qPCR. The results demonstrated that ICA significantly enhanced expression of FGFR1, FZD7 and DVL3, and significantly reduced the expression of GSK-3β. It was hypothesized that ICA-regulated expression of these genes presumably favored the proliferation of human NSCs.

In conclusion, NSCs derived from the corpus striatum of 16-20-week human fetuses obtained following spontaneous abortion were successfully isolated. The NSCs expressed nestin, an NSC marker, and could differentiate into various types of cells of the nervous system. Treatment with ICA promoted the proliferation and modulated gene expression in NSCs, thus suggesting that ICA may exert its neuroprotective effects by regulating NSC activity. Taken together, the findings suggest that ICA may be potentially applied for the treatment of neurodegenerative disorders.

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