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Ginsenoside Rg1 of *Panax ginseng* stimulates the proliferation, odontogenic/osteogenic differentiation and gene expression profiles of human dental pulp stem cells

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ABSTRACT

Ginsenoside Rg1 is one of the major active components of *Panax ginseng* C. A. Mey. Human dental pulp stem cells (hDPSCs) play an important role in the dentin formation, reparation and tooth tissue engineering. This study investigated the effects of ginsenoside Rg1 on the proliferation, odontogenic differentiation of hDPSCs and revealed the underlying molecular mechanisms. [^{3}H]-thymidine incorporation assay and cell cycle analysis were applied to investigate the proliferation of hDPSCs after the treatment of ginsenoside Rg1. Immunocytochemistry analysis and fluorescent quantitative reverse transcriptase-polymerase chain reaction (FQ-PCR) were performed to evaluate the odontogenic differentiation of hDPSCs. Gene and protein expressions of bone morphogenetic protein-2 (BMP-2) and fibroblast growth factor 2 (FGF2) were detected by FQ-PCR and enzyme-linked immunosorbent assay. The Roche Nimblegen Whole Human Genome Expression profile microarray was used to detected representative gene expression profiles of hDPSCs by ginsenoside Rg1. The results indicated that ginsenoside Rg1 significantly increased hDPSCs proliferation ($p < 0.05$). Gene expressions of DSPP, ALP, OCN, BMP-2, FGF2 and protein expressions of BMP-2 and FGF2 were increased compared with the untreated group ($p < 0.05$). Gene expression profile analysis revealed that 2059 differentially expressed genes were detected by ginsenoside Rg1. Ginsenoside Rg1 promoted the proliferation and differentiation of hDPSCs through alteration of gene expression profiles.

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Introduction

Dental pulp plays an important role in the reparative regeneration of tooth tissue. When a tooth injury, dental pulp is involved in reparative dentinogenesis and deposits a new dentin matrix to repair the injured site (Renjen et al. 2009). This is possible because of the presence of progenitor/stem cells in the dental pulp, which are able to form odontoblasts under appropriate microenvironments (Hao et al. 2004). Conventional root canal therapy depends on the removal of inflamed or necrotic pulp tissue and subsequent restoration with the insertion of a synthetic material into the root canal system, resulting in the loss of physiological form and function of the dental pulp (Bluteau et al. 2008). Complications such as root fractures can occur especially in teeth with incomplete root formation. Tissue engineering is leading to a great interest in biologic regeneration of damaged dental tissues. Regenerative dentin and pulp are expected to be potential tools for endodontic therapy, although technical problems need to be solved before they can be implemented in clinical applications (Cordeiro et al. 2008). Cells, scaffolds, and bioactive molecules are needed for tooth regeneration (Hung et al. 2011; Nakashima and Akamine 2005). Dental pulp tissue has been recently demonstrated to contain a population of postnatal stem cells (Gronthos et al. 2000, 2002). Gronthos et al.discovered dental pulp stem cells (DPSCs) with properties which are very similar to those of mesenchymal stem cells (MSCs). DPSCs possess stem cell-like properties, including self-renewal capability and multilineage differentiation. They can proliferate and differentiate into odontoblast-like cells under appropriate conditions (Gronthos et al. 2000). DPSCs are considered to be a potential source of cell-based therapy for developing a regenerative endodontic therapy and play an important role in tooth tissue engineering (Galler et al. 2011; Yamada et al. 2010).

Panax ginseng C. A. Mey is a tonic drug in traditional Chinese medicine and has been safely used in China for over 2000 years. Ginsenosides are the major active components of *Panax ginseng* C. A. Mey. Ginsenoside Rg1, abundant in *Panax ginseng* C. A. Mey, belongs to a family of steroids named steroidal saponins (Attele et al. 1999). It is one of the most active ingredients in *Panax ginseng*

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C. A. Mey and has a broad range of activities. Researches indicated that ginsenoside Rg1 could enhance bone marrow stromal cells and endothelial progenitor cells proliferation (Lu et al. 2008; Shi et al. 2009). Recent studies stated that ginsenosides Rg1 increased the number of osteoblasts, the activity of alkaline phosphatase (ALP) in cultured osteoblasts, increase bone formation while prevented ovariectomized rats bone loss (Gong et al. 2006; Shen et al. 2010). These findings indicated the potential use of ginsenoside Rg1 in endodontic biotherapy, reparative dentin formation and tooth tissue engineering.

To understand the behavior of ginsenoside Rg1 in dentin formation and reparation, in this study, we investigated the effects of ginsenoside Rg1 on DPSCs proliferation and differentiation into odontoblasts *in vitro*, and attempted to reveal the underlying mechanism by using microarray to identify the differentially expressed genes in DPSCs treated by ginsenoside Rg1.

Materials and methods

Cell culture

Fifty-nine healthy human impacted third molars were collected from adults (19–28 years old) in the Department of Oral and Maxillofacial surgery, the First Affiliated Hospital of Chongqing Medical University. The Human Ethical Committee of the First Affiliated Hospital of Chongqing Medical University approved our experimental protocols and informed consent was obtained from all subjects. DPSCs were isolated and cultured as previously described (Gronthos et al. 2000, 2002). Briefly, the pulp was separated from the crowns and roots, minced into small pieces, and then digested in a solution of 3 mg/ml collagenase type I (Sigma, USA) and 4 mg/ml dispase (Sigma, USA) for 30 min to one hour at 37 °C. Single-cell suspensions were obtained by passing these cells through a 70 μm strainer and cells were then cultured in 6-well plates (Costar, Corning, USA) at a density of 1×10^4 cells per well with alpha modification of Eagle's medium (α-MEM, Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA), 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma, USA) at 37 °C under 5% CO_2 condition. Culture medium was changed every 3 days. Cells at passages 3 or 4 were used for further experiments.

Proliferative ability assays

To investigate the proliferative ability of DPSCs after the treatment of different concentrations of ginsenoside Rg1 (0.1, 0.5, 2.5, 5, 10 and 20 μmol/l), DNA synthesis assay and flow cytometry analysis were applied. Ginsenoside Rg1 (purity ≥ 95%) was purchased from the Hongjiu biotechnology limited company (Jilin, China). The chemical structure of ginsenoside Rg1 is shown in Fig. 1.

DNA synthesis assay

DNA synthesis was investigated by [^{3}H]-thymidine incorporation assay (Balloni et al. 2009). DPSCs were seeded in 96-well plates containing growth medium at a density of 1×10^4 cells per well and cultured until 70–80% confluence. Then cells were treated with α-MEM medium containing 10% FBS with different concentrations of ginsenoside Rg1 (0.1, 0.5, 2.5, 5, 10, and 20 μmol/l) for 72 h. Control cultures received the same volume of culture medium without ginsenoside Rg1. During the last 8 h of incubation, 0.25 μCi/well of [^{3}H]-thymidine (Amersham Biosciences, Little Chalfont, United Kingdom) was added. [^{3}H]-thymidine incorporation was determined using a liquid scintillation counter (Wallac, Turku, Finland). The assay was performed in three independent experiments.

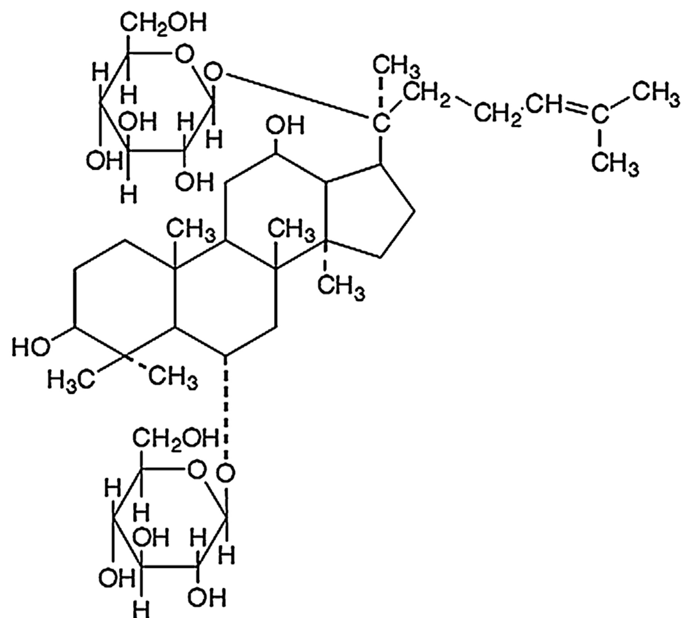

Fig. 1. The chemical structure of ginsenoside Rg1.

Cell cycle analysis

Cells were harvested after 3 days of being cultured in α-MEM medium containing 10% FBS and 5 μmol/l ginsenoside Rg1, and fixed in 70% ethanol at 4 °C overnight. Then cells were washed twice with 0.05 M PBS (pH 7.4), stained with propidium iodide (PI) at room temperature for 30 min, and analyzed by flow cytometry using CellQuest software for cell cycle analysis.

Odontogenic differentiation assays

To evaluate the odontogenic differentiation of DPSCs after the treatment of ginsenoside Rg1 at a concentration of 5 μmol/l, immunocytochemistry analysis of dentin sialoprotein (DSP) and FQ-PCR for gene expressions of dentin sialophosphoprotein (DSPP), alkaline phosphatase (ALP) and osteocalcin (OCN) were performed.

Immunocytochemistry analysis of dentin sialoprotein (DSP)

DPSCs were seeded in 24-well plates with cover slip on the bottom at a density of 1×10^4 cells per well and cultured in α-MEM medium containing 10% FBS and final concentration of ginsenoside Rg1 at 5 μmol/l. Controls were cultured in α-MEM with 10% FBS. The medium was changed every 3 days, and the cultures were maintained for 14 days. Cells were fixed with 4% formalin for 20 min, washed in phosphate-buffered saline (PBS), treated with 3% hydrogen peroxide for 10 min, washed and blocked with 5% normal goat serum for 30 min. Then specimens were incubated with primary antibodies (DSP) (Santa Cruz, 1:250 dilution) for 1 h at room temperature. The secondary antibody (biotinylated goat anti-mouse IgG) was added for 1 h at 37 °C. After washing, streptavidin–peroxidase complex was added and incubated for 30 min at 37 °C followed by washing and the addition of diaminobenzidine (DAB) staining solution for 5 min. Specimen were counterstained with Mayer's hematoxylin solution (Sigma, USA) and mounted with neutral resin. Normal saline instead of the primary antibody was served as the negative control.

Table 1
Primer sequences used in fluorescent quantitative RT-PCR.

Gene	Primers
DSPP	Forward: 5′ AATGCTGGAGCCACAAAC 3′
	Reverse: 5′ GCTTCCTTAGTCCCATTTC 3′
ALP	Forward: 5′ GAGCAGGAACAGAAGTTTGC 3′
	Reverse: 5′ GTTGCAGGGTCTGGAGAGTA 3′
OCN	Forward: 5′ CAAAGGTGCAGCCTTTGTGTC 3′
	Reverse: 5′ TCACAGTCCGGATTGAGCTCA 3′
BMP-2	Forward: 5′ GCCAGCCGAGCCAACAC 3′
	Reverse: 5′ AAATTAAAGAATCTCCGGGTTGT 3′
FGF2	Forward: 5′ CCCGACGGCCGAGTTGAC 3′
	Reverse: 5′ TTCATAGCCAGGTAACGGTTAGC 3′
GAPDH	Forward: 5′ AGTCCACTGGCGTCTTCA 3′
	Reverse: 5′ CGGACTTCTCATGGTTCACAC 3′

Gene expression analysis of DSPP, ALP and OCN

The gene expression of dentin sialophosphoprotein (DSPP), alkaline phosphatase (ALP) and osteocalcin (OCN) was analyzed using fluorescent quantitative reverse transcriptase-polymerase chain reaction (FQ-PCR). After DPSCs were incubated with different medium for 7, 14 and 21 days, respectively, total RNA was extracted from cells by using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA samples were reverse-transcribed into cDNA using a reverse-transcription kit. FQ-PCR was performed by using an ABI 7300 sequence detection system (Applied Biosystems, Foster City, CA) with SYBR Green reagent (Promega, USA). The primers for gene amplification were shown in Table 1. The reaction mixtures were incubated in a thermal cycle at 94 °C for 2 min, followed by 40 cycles at 94 °C for 15 s, 57 °C for 10 s (DSPP and OCN), or 58 °C for 15 s (ALP), 72 °C for 30 s, and a final extension at 72 °C for 2 min. Measured mRNA levels were normalized to the mRNA copies of GAPDH. All assays were repeated at least three times.

Determination the gene expression of bone morphogenetic protein-2 (BMP-2) and fibroblast growth factor 2 (FGF2)

The method of FQ-PCR to detect the gene expression of BMP-2 and FGF2 was previously described. After DPSCs were incubated with different medium for 3, 7 and 14 days, respectively, the samples were collected. The primers for gene amplification were also shown in Table 1. The reaction mixtures were incubated in a thermal cycle at 94 °C for 2 min, followed by 40 cycles at 94 °C for 15 s, 58 °C for 15 s, 72 °C for 30 s, and a final extension at 72 °C for 2 min. All assays were repeated at least three times.

Table 2
[^{3}H]-Thymidine incorporation assay.

Group	Concentrations of ginsenoside Rg1 (μmol/l)	[^{3}H]-TdR incorporation value
Control group	0	400.87 ± 9.17
Ginsenoside Rg1 group	0.1	409.61 ± 9.31
	0.5	891.38 ± 7.17[a]
	2.5	1045.35 ± 28.18[a]
	5	2023.29 ± 24.48[a b]
	10	1055.35 ± 33.91[a]
	20	417.04 ± 11.58

Data were presented as the means ± SD. $n = 6$.
[a] Compared with control group, $p < 0.05$.
[b] Compared with 0.5, 2.5 and 10 μmol/l ginsenoside Rg1 group, $p < 0.05$. One-way analysis of variance and multiple comparisons [SNK-q test].

Determination the protein expression of bone morphogenetic protein-2 (BMP-2) and fibroblast growth factor 2 (FGF2)

DPSCs (1×10^4 cells/well) were seeded in 24-well plates and cultured for 24 h. The cells were treated with α-MEM medium containing 10% FBS with 5 μmol/l ginsenoside Rg1. Medium without ginsenoside Rg1 served as a control. After 3, 7, and 14 days of incubation, culture supernatant was collected and stored at −20 °C. The amounts of BMP-2 and FGF2 were determined by enzyme-linked immunosorbent assay (ELISA) kits (R&D System, Minneapolis, USA) according to the manufacturers' instructions. The assay was performed in three independent experiments.

Gene expression profile microarray analysis

Gene expression in the experimental group and control group of DPSCs was investigated with the Roche Nimblegen Whole Human Genome Expression profile microarray (Roche Nimblegen, USA) system consisting of 44,049 genes. Total RNA was extracted from cells by using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA quantity and purity were determined using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and denaturing gel electrophoresis. Microarray experiment was performed in triplicate according to the manufacture protocol. In brief, total RNA samples (5 mg) were amplified and reverse-transcribed into cDNA using a reverse-transcription kit. The synthesized cDNA was labeled with Cy3 dye using a NimbleGen One-Color DNA Labeling Kit. After purification, the labeled cDNA was applied to a Roche Nimblegen Whole Human Genome Expression profile microarray (Roche Nimblegen, USA) and then hybridized in NimbleGen

Table 3
Effect of ginsenoside Rg1 on cell cycle distribution of DPSCs.

Group	G_0/G_1 phase	G_2/M phase	S phase	PrI(S + G_2/M)
Control group	85.74 ± 2.45	7.36 ± 1.80	6.90 ± 2.43	14.26 ± 2.44
Ginsenoside Rg1 group	65.13 ± 1.47[a]	13.67 ± 0.96[a]	21.20 ± 2.11[a]	34.87 ± 1.47[a]

Data were presented as the means ± SD. $n = 6$.
[a] Compared with control group, $p < 0.05$. Multivariate analysis of variance.

Table 4
Gene ontology analysis.

Gene ontology: biological process terms	Genes	*p*-Value
Cell cycle	CCNB1, CCNB2, MNAT1, CDC2, CDKN1A, CDKN3, CDKN2A, ANAPC1, CDK10	2.7 E−14
Cellular metabolism	KRT15, KIF1B, ZNF697, PRG2, P2RY2, IMMP2L	4.3E−13
Growth factor and growth factor receptor activity	BMP-2, TGFβ1, VEGF, BMP8B, CSF1, FGFR1, BMPR2, IGF2R, PDGFB, TGFBR2, NRP1, CCR2	1.2E−11
Biosynthetic process	CEL, COL11A2, ACPP, MMP14, CACNB1, ALPL, CDH1, ITGA3, SERPINB10, TAF4B, ABCB10, IRF8	1.8E−9
Cell proliferation	ATF3, MKI67, S100A6, FTH1, DHCR7	2.1E−8
Signal transduction	MAP2K3, MAPK14, MAP3K10, BAMBI, NDRG2, ECM1, SMAD7	5.5E−7
Apoptosis	MYC, P53AIP1, ZBTB16, BBC3, VHL, CASP3, APITD1	1.3E−5

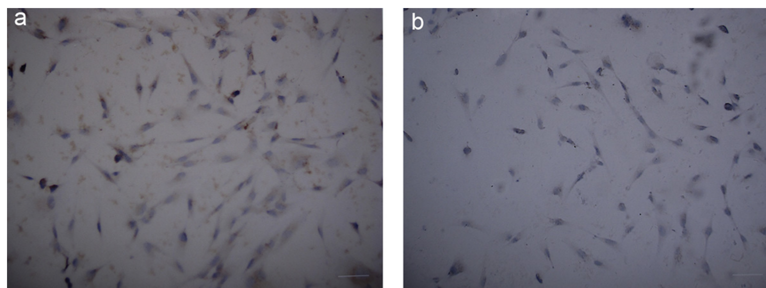

Fig. 2. Immunocytochemical stain of DSP expression in ginsenoside Rg1 treated DPSCs (a) and control group (b). Scale bar = 200 μm.

Hybridization System at 60 °C for 17 h. After hybridization and washing, the microarray was scanned on Axon GenePix 4000B microarray scanner (Molecular Devices, USA). Then the scanned image was analyzed and normalized using NimbleScan software. The standard to judge the differentially expressed genes was the ratio of the experimental group and control group ≥2. The differentially expressed genes were classified with gene ontology (GO) analysis according to their functions. Pathway analysis was used to select the statistically significant pathways. The gene expression profiling data complied with the Minimum Information About Microarray Experiments (MIAME) standard (Brazma et al. 2001).

Statistical analysis

The quantitative data were presented as means ± standard deviation. The results were analyzed by one-way analysis of variance (analysis for DNA Synthesis Assay), factorial design analysis of variance (analysis for ELISA and FQ-PCR), multivariate analysis of variance (analysis for cell cycle) and multiple comparisons (SNK-q test). Statistical analysis was performed using SPSS statistical software (version 12.0, SPSS Inc., Chicago, IL, USA). Values of $p < 0.05$ were considered to be statistically significant.

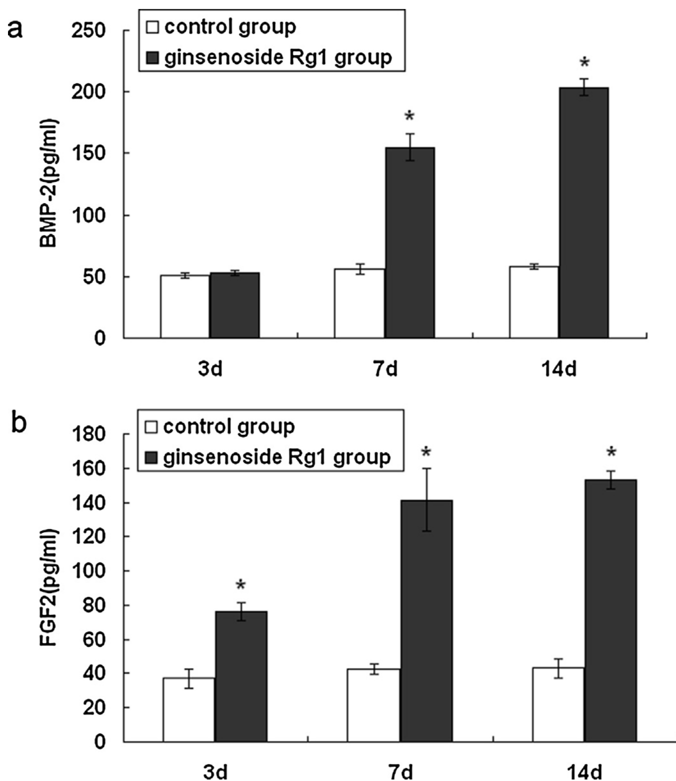

Fig. 3. Ginsenoside Rg1 induced BMP-2 (a) and FGF2 (b) expressions in DPSCs. BMP-2 and FGF2 productions were determined by enzyme-linked immunosorbent assay method of DPSCs culture supernatants after 3, 7, and 14 days incubation with 5 μmol/ö ginsenoside Rg1. Ginsenoside Rg1 significantly enhanced BMP-2 expression at day 7 and day 14 compared to the untreated group (a). Ginsenoside Rg1 significantly enhanced FGF2 expression at day 3, day 7 and day 14 compared to the untreated group (b). Data were presented as the means ± SD $n = 6$. *Compared with control group, $p < 0.05$. Factorial design analysis of variance and multiple comparisons (SNK-q test).

Results

DNA synthesis assay

The [^{3}H]-thymidine incorporation assay was used to demonstrate DPSCs proliferation after 72 h incubation with varying concentrations of ginsenoside Rg1 (Table 2). Comparing with the control group, ginsenoside Rg1 significantly promoted the proliferation of DPSCs at concentrations of 0.5, 2.5, 5, and 10 μmol/l, especially 5 μmol/l ($p < 0.05$).

Cell cycle analysis

The effect of ginsenoside Rg1 on cell cycle distribution of DPSCs was determined by flow cytometry (Table 3). It was shown that 5 μmol/l ginsenoside Rg1 significantly increased the proportion of cells in the proliferative phase (S phase) while decreased the cells in the resting phase (G_0/G_1 phase) ($p < 0.05$). The proliferation index (PrI) was also advanced compared with control group ($p < 0.05$).

Immunocytochemistry analysis of DSP

After 14 days of ginsenoside Rg1 treatment on DPSCs, odontogenic differentiation of DPSCs was shown by the positive immunostaining of DSP in ginsenoside Rg1 group (Fig. 2).

Protein expression of bone morphogenetic protein-2 (BMP-2) and fibroblast growth factor 2 (FGF2)

BMP-2 and FGF2 secretions were evaluated by incubating DPSCs in the presence of 5 μmol/l ginsenoside Rg1. The culture supernatants were collected for quantification of protein level at 3, 7 and 14 days, respectively. At day 7 of incubation, ginsenoside Rg1 significantly enhanced BMP-2 expression by around 2.7 times compared with the untreated group. At days 14 of incubation, ginsenoside Rg1 significantly stimulated BMP-2 expression by 3.5 times compared with the untreated group ($p < 0.05$) (Fig. 3a). At 3, 7 and 14 days, ginsenoside Rg1 significantly stimulated FGF2 secretions by 2 times, 3 times and 3.5 times compared with the untreated group, respectively ($p < 0.05$) (Fig. 3b).

Expression of DSPP, ALP, OCN, BPM-2 and FGF2 mRNA

The expressions of DSPP, ALP, OCN, BMP-2 and FGF2 mRNA in the ginsenoside Rg1 group (5 μmol/l) showed a time-dependent

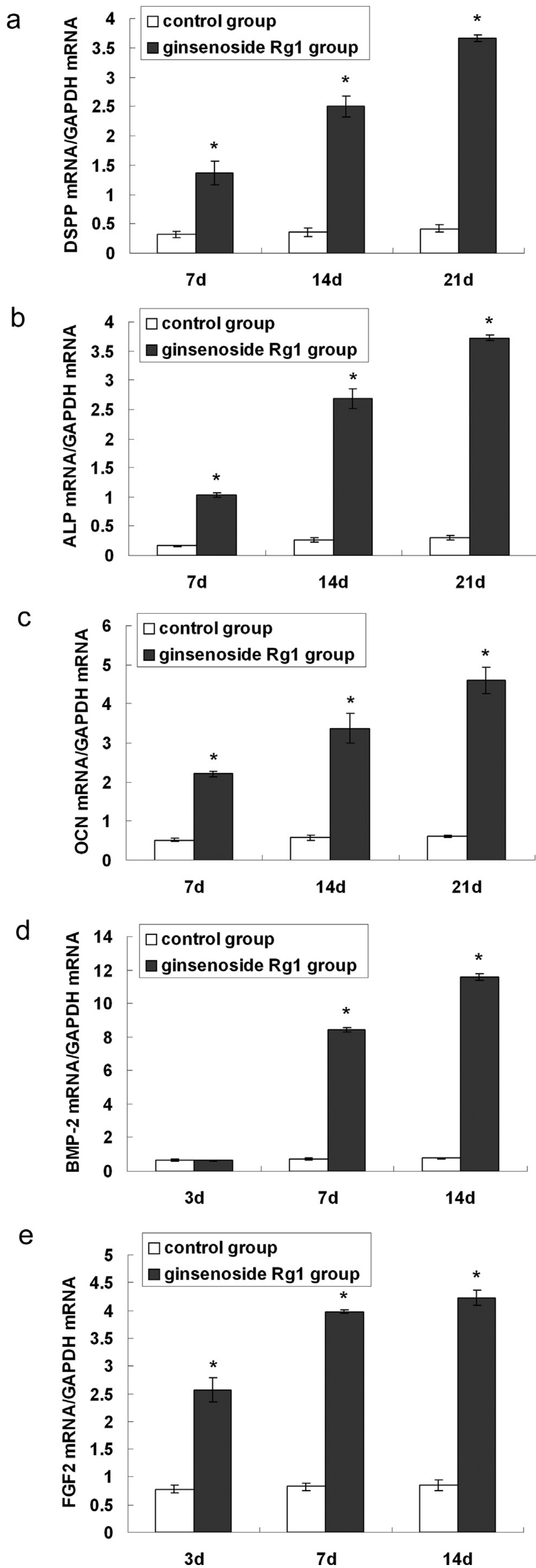

Fig. 4. FQ-PCR analysis of the gene expressions after ginsenoside Rg1 treatment on DPSCs. DPSCs were cultured in the control group and ginsenoside Rg1 group (5 μmol/l) for the indicated times. Total RNA was isolated, whilst DSPP, ALP, OCN, BMP-2 and FGF2 gene expressions were determined by FQ-PCR. Ginsenoside Rg1 significantly enhanced the DSPP mRNA (a), ALP mRNA (b), OCN mRNA(c), BMP-2 mRNA (d) and FGF2 mRNA (e) levels. Data were shown as the means ± SD of three independent experiments $n = 3$. *Compared with control group, $p < 0.05$. Factorial design analysis of variance and multiple comparisons (SNK-q test).

Table 5
KEGG pathway analysis.

Pathway ID	KEGG Pathway terms	*p*-Value
hsa04110	Cell cycle	2.43531E−7
hsa04010	MAPK signal pathway	6.34004E−6
hsa04350	TGF-beta signaling pathway	5.33899E−5
hsa04115	p53 signaling pathway	2.70418E−4
hsa04512	ECM-receptor interaction	0.01031297
hsa05120	Epithelial cell signaling in Helicobacter pylori infection	0.01292882
hsa04062	Chemokine signaling pathway	0.03882413

increase compared with the control group ($p < 0.05$), which remained little change in the period (Fig. 4).

Gene expression profile microarray analysis

The Roche Nimblegen Whole Human Genome Expression profile microarray was used to compare representative gene expression profiles of DPSCs in the ginsenoside Rg1 (5 μmol/l) group and control group. The results indicated that 2059 differentially expressed genes out of 44,049 genes were detected between the ginsenoside Rg1 (5 μmol/l) group and control group of 2.0-fold or more. There were 1498 up-regulated genes and 561 down-regulated genes in the ginsenoside Rg1 (5 μmol/l) group of 2.0-fold or more. The top thirty up-regulated genes and down-regulated genes were listed in Supplementary Tables 1 and 2, respectively. The most statistically significantly represented GO terms were displayed in Table 4. Gene ontology categories showed that these genes were mainly associated with cell cycle, cellular metabolism, biosynthetic process, signal transduction, growth factor, cell proliferation and apoptosis of functional gene categories. Table 5 shows the statistically enriched KEGG pathway terms for differentially expressed genes. Pathway analysis found seven statistically significant pathways including cell cycle pathway, MAPK signal pathway and TGF β signal pathway ($p < 0.05$).

Discussion

Stem cell biology has become an important field for the understanding of tissue regeneration and implementation of regenerative medicine. Recent advances in stem cell biology have revealed that progenitor cells which are named DPSCs are also present in dental pulp tissue (Gronthos et al. 2000; Miura et al. 2003). DPSCs possess postnatal stem cell characteristics, including multipotent differentiation, self-renewal, clonogenic capacity, and expression of multiple mesenchymal stem cell surface markers (Gronthos et al. 2000, 2002; Shi and Gronthos 2003). DPSCs have gained great importance for use in the regenerative treatment of defective dental tissues, particularly those in the dentin-pulp complex when transplanted into immunocompromised mice by using HA/TCP as a carrier (Yang et al. 2008; Zhang et al. 2008). Therefore, DPSCs are considered to be suitable cells to evaluate odontogenic differentiation both *in vitro* and *in vivo*.

Recent reports state that *Panax ginseng* C. A. Mey and its constituents have anti-neoplastic, anti-oxidation, anti-inflammation, and estrogen-like activities (Attele et al. 1999; Lau et al. 2008; Zhu et al. 2009). Ginsenoside Rg1 promoted cells proliferation, differentiation and bone formation (Gong et al. 2006; Lu et al. 2008; Lu et al. 2009; Shen et al. 2010; Shi et al. 2009).

In this study, the effects of ginsenoside Rg1 on the proliferation and odontogenic differentiation of DPSCs were investigated. Our data showed that ginsenoside Rg1 significantly stimulated DPSCs proliferation and promoted the odontoblast differentiation. Ginsenoside Rg1 induced the production of DSP. The level of DSPP, ALP, and OCN mRNAs increased in a time dependent manner. DSPP, ALP, OCN, and DSP have been reported as mineralization markers for odontogenic/osteogenic differentiation (Jittapiromsak et al. 2010; Lee et al. 2010). The enhanced ALP activity is present as a marker during the early differentiation phase and plays an important role in mineral deposition (Fiorentini et al. 2011; Park et al. 2009). DSP is a dentin extracellular matrix protein that functions as an initiator of mineralization. DSP production is widely recognized as one of the most specific markers of the odontoblast phenotype. DSP and dentin phosphoprotein (DPP) play important roles in extracellular matrix mineralization and dentinogenesis. DSPP is a dentin extracellular matrix protein that functions as an initiator of mineralization and considered one of the specifically odontoblastic markers (Lee et al. 2010; Suzuki et al. 2009). Osteocalcin, a vitamin K-dependent noncollagenous extracellular matrix protein, is synthesized by osteoblasts and odontoblasts. OCN is frequently used as a marker of odontogenic or osteogenic differentiation. The up-regulation of the dentin-specific genes DSPP, ALP, and OCN in treated DPSCs demonstrates the potential role of ginsenoside Rg1 in dentin regeneration and tooth engineering in conjunction with DPSCs.

BMP-2 plays an important role in tooth and bone formation. This protein has been successful in inducing bone and dentin formation both *in vitro* and *in vivo* studies (Ikeda et al. 2011; Yang et al. 2009). BMP-2 promotes odontoblast differentiation and mineral deposition. It accelerates DSPP mRNA expression, but does not influence cell proliferation in human pulp cells (Saito et al. 2004). In the present study, ginsenoside Rg1 increased BMP-2 mRNA levels in DPSCs, which may suggest that BMP-2 promoted the odontogentic differentiation of DPSCs and DSPP expression.

Ginsenoside Rg1 is a kind of potent phytoestrogen which can stimulate bone marrow stromal cells proliferation *via* the activation of the estrogen receptor-mediated signaling pathway (Lu et al. 2008). Estrogens can activate BMP-2 gene transcription in mouse mesenchymal stem cells and stimulate MSCs differentiation into osteoblasts (Zhou et al. 2003). Since DPSCs possess mesenchymal stem cell characteristics, it might be possible that estrogen receptor-mediated signaling pathway is involved in promoting DPSCs proliferation and differentiation resulted from ginsenoside Rg1 treatment.

FGF is involved in self-renewal of MSCs and the maintenance of their multilineage differentiation potential (Tsutsumi et al. 2001; Yeoh and Haan 2007). It has been reported that FGF2 as a cytokine can exert a significant effect *in vitro* on hDPSCs proliferation and control extracellular matrix generation during tissue generation and wound healing (He et al. 2008; Nakao et al. 2004). In the present study, FGF2 mRNA and protein were detected in DPSCs treated by ginsenoside Rg1.

In the present study, representative gene expression profiles and functional classifications were compared between the ginsenoside Rg1 and control group on hDPSCs by using a cDNA microarray system and a clustering algorithm. 2059 differentially expressed genes were detected between the ginsenoside Rg1 (5 μmol/l) group and control group (1498 up-regulated genes and 561 down-regulated genes). Gene ontology analysis of the differentially expressed genes showed these genes mainly related to the following functional gene categories: cell cycle, cellular metabolism, growth factor and growth factor receptor activity, biosynthetic process, cell proliferation, signal transduction and apoptosis. These genes may play important role in DPSCs proliferation and differentiation. Pathway analysis found seven statistically significant pathways such as cell cycle pathway, MAPK signal pathway and TGFβ signal pathway. The TGFβ family plays a crucial role in regulating the proliferation, extracellular matrix formation, differentiation, migration, and apoptosis of cells (Chan et al. 2005; Lee et al. 2006).

This *in vitro* study examined the effects of ginsenoside Rg1 on DPSCs with an attempt to clarify whether the Chinese medicine has a potential of DPSCs proliferation and odontogenic differentiation. The results demonstrated that ginsenoside Rg1 promoted the proliferation and differentiation of DPSCs into odontoblast-like cells by regulating the expression of a series of related genes and pathways. The data presented here indicate that ginsenoside Rg1 may be used in endodontic biotherapy, reparative dentin formation and tooth tissue engineering. This investigation is a first step toward that long-term goal of biological regenerative endodontic therapy and tooth tissue engineering.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phymed.2013.08.021.

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