



Ginsenoside Re promotes proliferation of murine bone marrow mesenchymal stem cells in vitro through estrogen-like action

Linzi Luo¹ · Bin Peng² · Lei Xiong² · Baohe Wang² · Linghao Wang³

Received: 9 May 2024 / Accepted: 9 August 2024 / Editor: Tetsuji Okamoto
© The Society for In Vitro Biology 2024

Abstract

Ginsenoside Re (GS-Re) is a major saponin monomer found in *Panax ginseng* Meyer. It has been shown to exhibit a wide range of biological and pharmacological activities. This study aimed to investigate the effect of GS-Re on the proliferation of murine bone marrow-derived MSCs in vitro and to assess whether its effect is dependent on the estrogen receptor-mediated signal transduction. CFU colony formation assay, cell counting, and colorimetric MTT test were employed to examine effects of GS-Re on the in vitro proliferation of MSCs and the mechanisms of the underlying effect were detected by flow cytometric analysis, immunofluorescence staining for BrdU, and Western blotting. GS-Re dose-dependently promoted the in vitro proliferation of murine bone marrow-derived MSCs over a range of concentrations of 0.5–20 $\mu\text{mol/L}$, and this effect approached the maximal level at 10 $\mu\text{mol/L}$. Increases in the expression level of phosphorylated extracellular signal-regulated kinases 1/2 (p-ERK1/2) were observed in the passaged MSCs treated with 10 $\mu\text{mol/L}$ of GS-Re. These effects of GS-Re on the MSCs were significantly counteracted by the addition of ICI 182, 780 (an estrogen receptor antagonist) to the culture media. We concluded that GS-Re is able to exert a proliferation-promoting effect on murine bone marrow-derived mesenchymal stem cells in vitro, and its action is involved in the estrogen receptor-mediated signaling.

Keywords Ginsenoside Re · Mesenchymal stem cells · Proliferation · Estrogen-like activity

Introduction

The ginsenoside is a big family of triterpenoid saponins from *Panax ginseng* C.A. Meyer. Ginseng, the root of *Panax ginseng*, has been used as a kind of valuable phytomedicine in Oriental countries with a very long history. Nowadays, there also is a growing use of ginseng products as a functional food and alternative medicine around the world (Shi *et al.* 2019; Bilia and Bergonzi 2020). Ginsenosides have been shown to be the main bioactive ingredient in *Panax ginseng*. They are not only distributed in roots of various *Panax* species, but also in other parts of the plant, including

the leaf, stem, flower head, and berry. Up until now, more than 170 naturally occurring ginsenosides have been isolated and identified, and they, with rare exceptions, share a similar basic structure, consisting of a saturated sterane steroid nucleus (Piao *et al.* 2020; Zhang *et al.* 2021). Most of the ginsenosides can be divided into two classes according to the skeleton of aglycones, namely dammarane-type and oleanane-type. The dammarane-type saponins, mainly comprising protopanaxadiol (PPD) and protopanaxatriol (PPT) groups, account for the overwhelming majority of the ginseng total saponins (Christensen 2009; Piao *et al.* 2020). Ginsenoside Re (G-Re), a saponin monomer, is a major member of the protopanaxatriol group. It occurs abundantly in roots of *Panax ginseng*, and its abundance is higher in berries, flower buds, and leaves of this plant (Kim *et al.* 2009). G-Re has been documented to exhibit a variety of biological and pharmacological activities in vivo and in vitro, such as anti-diabetes, anti-Parkinson's disease, anti-Alzheimer's disease, anti-oxidant, anti-cancer, cardiovascular protection, and immunomodulatory effects (Lim *et al.* 2013; Gao *et al.* 2022).

✉ Linghao Wang
linghaowang@126.com

¹ Department of Endoscopic Diagnosis and Treatment, Hunan Chest Hospital, Changsha, China

² School of Life Science, Hunan Normal University, Changsha, China

³ Department of Endocrinology and Metabolism, Third Xiangya Hospital of Central South University, Changsha, China

Mesenchymal stem cells, or multipotent mesenchymal stromal cells (MSCs), are defined as plastic-adherent, fibroblast-like cells which possess a proliferative potential and a capacity to differentiate into chondrogenic, osteogenic, adipogenic, and probably other cell lineages *in vitro* (Pittenger *et al.* 1999; Bhat *et al.* 2021). Functionally, MSCs can secrete a wide array of growth factors and cytokines and release extracellular vesicles which comprise the components of extracellular matrix and the various types of bioactive molecules. These compounds are able to exert the multifaceted biological activities, including the maintenance of tissue homeostasis, the regulation of biological function, and the improvement of immune response (Haynesworth *et al.* 1996; Deng *et al.* 2018). MSCs were first isolated from bone marrow stroma and they were initially described as colony-forming unit fibroblasts (CFU-Fs). Although the bone marrow remains the main source for obtaining MSCs, these stem cells can also be isolated from almost every organ in the body (Friedenstein *et al.* 1970; Ferrin *et al.* 2017; Yosupov *et al.* 2017). The supernatant or extracellular vesicles derived from the *in vitro* culture of MSCs have indicated the therapeutic effects in disease or injury models and clinical trials. These observations have led to suggest that MSCs would maintain the homeostasis of the tissue in where they reside. At present, it is believed that MSCs do not only contribute structurally to tissue repair by the replenishment of cells but also favor the regeneration of damaged tissues by the modulation of the local microenvironment (Levy *et al.* 2019; Hade *et al.* 2021).

The high proliferative potential is a fundamental property of stem and progenitor cells. MSCs are able to massively propagate *in vitro*; however, their expansion has a limitation over a culture period. After primary MSCs have divided for a few dozen generations, their descendant cells will enter replicative senescence and ultimately cease proliferating during subculture (Wagner *et al.* 2008). On the other hand, CFU-F colony-forming and *in vitro* cell expansion assays suggested that the *in vivo* proliferative ability of MSCs in tissue or organs would decrease under certain physiological and pathophysiological conditions. It has been shown that the decreased proliferative capacity of MSCs is generally accompanied by their impaired differentiation, migration, and secretion (Siegel *et al.* 2013; Liu *et al.* 2017). Thus, the maintenance for the proliferative potential of MSCs is essential to their sufficient expansion *in vitro* and normal functioning *in vivo*. Over the past decade, seeking for the proper ways or agents facilitating the proliferation of MSCs has become an interesting topic in the relevant research fields of biology and medicine (Sun *et al.* 2011; Zanichelli *et al.* 2012).

In recent years, considerable attention has been paid to whether ginsenosides can exert their effects by modulating biological activities of MSCs. The effects of several kinds of

ginsenoside monomers, such as G-Rg1, G-Rg3, and G-Rb1, on the proliferation, differentiation, aging, and apoptosis of MSCs have been described in a number of papers (Hong *et al.* 2020; Liu *et al.* 2022; Xiao *et al.* 2022). Furthermore, the molecular mechanisms underlying the action of ginsenosides on MSCs have been understood in some ways, including the modulation of multiple signaling cascades and the alteration of gene expression profiles (Mohanani *et al.* 2018). Since most known ginsenosides share a rigid tetracyclic steroidal carbon skeleton which makes them suitable candidates to interact and activate steroid receptors, such as glucocorticoid and estrogen receptors, steroid hormone-like activities of different ginsenosides were assayed in multiple cell types (Bae *et al.* 2005). A group of researchers reported that G-Rg1 enhanced the proliferation of MSCs via the estrogen receptor-mediated signaling pathway (Lu *et al.* 2008).

To date, there has been little research into the biological activity of G-Re in MSCs. The present study was conducted to examine the effects of G-Re on the proliferation of MSCs isolated from murine bone marrow and to assess the dependence of its action on the involvement of estrogen receptors.

Materials and methods

Preparation of G-Re stock solution Ginsenoside Re (G-Re, purity > 98%) was obtained from the Department of Natural Medicinal Chemistry, School of Pharmaceutical Sciences, Jilin University, Changchun, Jilin, China. Stock solution of G-Re (250 $\mu\text{mol/L}$) was temporarily prepared before use. G-Re was dissolved in phenol red-free Dulbecco's modified Eagle's medium (PR-free DMEM; Gibco, Baltimore, MD) by utilizing the ethanol as a hydrotropy agent, the concentration of which is 1.25% (V/V) in the stock solution.

Cell isolation and culture SPF Kunming mice (female or male, 4–6 wk, body weight 18–22 g, approval number: SCXK-2013-0006) served as a cell source. The bone marrow was harvested by flushing the cavity of femurs and tibias of the mice with DMEM, and prepared into the single cell suspension. The nucleated bone marrow cells (NBMCs) were counted. The isolated bone marrow cells were used for the colony-forming unit fibroblast (CFU-F) assays and the expansion cultures of MSCs. The cells were tested and authenticated to be mesenchymal stem cells using flow cytometry. Both primary and subcultured cells were incubated at 37 °C and 10% CO₂ in DMEM containing 20% inactivated fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin (Invitrogen, Carlsbad, CA), unless otherwise specified.

CFU-F assay The freshly isolated bone marrow cells were seeded in 24 well plates at a density of 1×10^5 NBMCs/cm².

After the cultures had been incubated for 48 h, media and non-adherent cells were discarded, and fresh media in the presence or absence of G-Re at different concentrations were added. That is to say, the cultured cells were divided into six groups: five G-Re treatment groups (0.5, 1.0, 5.0, 10.0, and 20.0 $\mu\text{mol/L}$, respectively) and a vehicle control. Subsequently, the cells were cultured for a period of 7 d during which half-volume media changes were performed every 2 d to maintain relatively stable levels of medium compositions. At the end of the culture period, the cultures were rinsed three times with phosphate-buffered saline (PBS, Gibco, Gaithersburg, MD) and stained with Wright-Giemsa dye. CFU-F colonies were examined and photographed microscopically. A cellular aggregation containing > 20 fibroblast-like cells counted as a colony. The frequency of CFU-F colonies was calculated as the number of counted CFU-F colonies per 1×10^6 plated NBMCs. Colony size was measured using a calibrated graticule, and represented as mean area of the 10 largest colonies per well ($\text{mm}^2 \pm \text{SD}$).

Cell proliferation assay The third passage MSCs were cultured in 96-well plates with 3×10^3 cells/well. Following a 24-h attachment period, non-adherent cells were removed, and the remaining cells were grouped the same as that in CFU-F assays. After the cells were cultured for 48 h, the cells in plates were rinsed three times with PBS and stained with Wright-Giemsa dye. The cells in 10 randomly selected microscopic fields were counted per culture well. The total number of cells per well was calculated by multiplying the average number of cells/field by the ratio of total area of well/area of field.

Cell viability assay Methylthiazolyldiphenyl-tetrazolium bromide (MTT, Sigma, St. Louis, MO) assay was employed to evaluate the viability of culture-expanded MSCs. The third passage MSCs were seeded in 96-well plates at a density of 10^4 cells per well. Twenty-four hours later, the media were changed to divide the cultures into six groups as mentioned above. The cells were incubated for 48 h. MTT solution was added to each well and the cells were subsequently incubated for 4 h. After the culture supernatant was removed, the formazan crystals formed in intact cells were dissolved by adding dimethyl sulfoxide (DMSO) solution. The plates were shaken for 10 min to fully solubilize the formazan in a dark place. The absorbance was measured at 490 nm with a reference at 630 nm using an ELx 800 microplate reader (Bio-Tek Instruments, Winooski VT).

Blockade of estrogen receptor ICI 182, 780, an estrogen receptor antagonist, (MedChemExpress, South Brunswick, NJ) was used for the blockade assay of estrogen receptor. Both primary bone marrow cells and the third-generation MSCs were incubated for 48 and 24 h, respectively,

and non-adherent cells were removed. The adherent cells were treated with G-Re (10 $\mu\text{mol/L}$) or 17- β estradiol (E2, 10^{-8} mol/L, Sigma-Aldrich, St. Louis, MO) in the presence and absence of ICI 182, 780 (10^{-6} mol/L), and with ICI 182, 780 (10^{-6} mol/L) alone or vehicle alone. That is to say, these two kinds of cells were divided into six groups denoted as Re, Re + ICI, E2, E2 + ICI, ICI, and VC, respectively. CFU-F and cell proliferation assays were conducted as previously described to examine the influence of estrogen receptor blockade on the cell growth.

Cell cycle phase analysis MSCs at passage 3 were seeded at the density of 2×10^5 cells/ cm^2 in 25- cm^2 culture flasks and 24 h later non-adherent cells were discarded. The adherent cells were divided into six groups: Re, Re + ICI, ICI, E2, E2 + ICI, and VC as mentioned above. After another 24 h of culture, the cells were harvested by trypsinization, and then washed twice with PBS. Subsequently, the cells were suspended in PBS and fixed in 70% ethanol at 4 °C overnight. The ethanol-fixed cells were collected by centrifugation. The obtained cells were washed twice with PBS and incubated in a staining solution containing propidium iodide, RNase A, and Triton X-100 for 30 min in the dark at room temperature. For each cell population, DNA contents of at least 10,000 cells were measured using a flow cytometer (Accuri C6, BD Biosciences, San Jose, CA). Quantitative analysis of cell cycle phase distribution was performed using ModFit LT software.

BrdU immunofluorescence staining The third passage MSCs were grown on the poly-L-lysine-coated glass coverslips in 35-mm Petri dishes to reach sub-confluent state, and then serum-starved to synchronize the cell cycle at G0/G1 phases in 24 h. After serum-free media were replaced by normal media, the cultures were divided into the aforesaid six groups: Re, Re + ICI, ICI, E2, E2 + ICI, and VC and subsequently incubated for an additional 24 h. At this time, the 5-bromo-2'-deoxyuridine (BrdU, Boster Biological Technology Co., Ltd, Wuhan, China) was added to medium in every group at final concentration of 30 $\mu\text{g/L}$ and the cells were incubated overnight. BrdU incorporation was detected with a mouse anti-BrdU primary antibody and a cyanine-conjugated secondary antibody (Boster Biological Technology Co., Ltd). The cells were counterstained with Hoechst 33342 (Sigma-Aldrich) and then photographed under a fluorescence microscope. The number of BrdU-positive nuclei and the total number of nuclei were counted in at least 10 randomly selected fields per dish. Value of positive nuclei in all dishes was expressed as a percentage of the total number of nuclei in the same dishes for every experimental group.

Western blotting The third passage MSCs were seeded at the density of 10×10^6 cells/dish in 10-cm Petri dishes.

After a 24-h incubation period, non-adherent cells were discarded and the adherent cells were divided into the aforesaid six groups: Re, Re + ICI, ICI, E₂, E₂ + ICI, and VC. After another 24 h of incubation, the cells were washed with cold $1 \times$ PBS and lysed with cell lysis buffer. The lysates were centrifuged at $12,000 \times g$ for 10 min at 4°C to obtain supernatants. The protein concentrations were measured by using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) and adjusted to become equal to each other between all samples. Equivalent amounts of protein were run in 10% SDS–polyacrylamide gel, and electrotransferred to a nitrocellulose membrane. The membranes were blocked with 5% fat-free milk in tris-buffered saline with 0.1% Triton X-100 (TBST) at 4 °C overnight, and then incubated with the primary antibody (rabbit anti-phospho-ERK1/2, Cell Signaling Technology, Danvers, MA) followed by horseradish peroxidase (HRP)–conjugated secondary antibody (goat anti-rabbit IgG, Abcam, Cambridge, UK), at room temperature for 3 h and 2 h, respectively. Protein bands specifically bound by antibodies were visualized using an ECL Western Blotting Substrate kit (Pierce, Thermo Fisher Scientific, Waltham, MA). Relative intensities of these bands were evaluated by Quality One analysis software (Bio-Rad Laboratories) and glyceraldehyde-3-phosphate dehydrogenase was used as an internal control. The results were expressed as the fold-changes over vehicle control (VC).

Data analysis All of the data are presented as mean \pm standard deviation (SD). The results were obtained from four independent experiments for CFU-F efficiency, cell proliferation, and cell viability assays, and from three independent experiments for Western blotting and BrdU incorporation assay. Statistical comparison of these results was performed by use of one-way ANOVA with the Fisher's least significant difference post hoc test at a significance level of $p < 0.05$. Cell cycle phase analysis was independently carried out three times; Pearson's chi-square test for the $R \times C$ contingency tables with Bonferroni's correction for multiple comparisons was applied to analyze the data at the overall significance level of 0.05 and Bonferroni corrected level of 0.003. PASW Statistics version 18.0 (SPSS Inc., Chicago, IL) was used for all statistical analyses.

Results

Ginsenoside Re promotes the proliferation of MSCs in a dose-dependent manner CFU-F assay was performed on primary bone marrow cells to evaluate the effect of G-Re on the colony-forming capacity of MSCs. As shown in Fig. 1, the size and yield of CFU-F colonies were increased by raising G-Re concentration in a range from 0.5 to 20 $\mu\text{mol/L}$. In comparison with the control, the increases in the colony

area were statistically significant at 5, 10, and 20 $\mu\text{mol/L}$ of G-Re. The yield of CFU-F colonies in G-Re-treated and control groups registered as 20.90 ± 4.14 , 22.17 ± 3.88 , 24.02 ± 5.05 , 26.40 ± 4.16 , 26.82 ± 2.58 , and 20.07 ± 2.82 per 10^6 NBMCs, respectively. CFU-F efficiencies at 10 and 20 $\mu\text{mol/L}$ of G-Re were significantly higher than the control value.

The effects of G-Re on the proliferation of culture-expanded MSCs were examined by means of direct cell counting and MTT assay. The results are shown in Fig. 2. The numbers of expanded MSCs increased gradually following the rise in G-Re concentration. The increases in cell counts of G-Re groups at 1, 5, 10, and 20 $\mu\text{mol/L}$ were statistically significant compared with the control group. There were significant differences of MTT reading between the group exposed to G-Re at 5, 10, or 20 $\mu\text{mol/L}$ and the control group.

Ginsenoside Re enhances the proliferation of MSCs by exerting its estrogen-like effect The CFU-F assay of primary bone marrow cells and the proliferation test of culture-expanded MSCs were also conducted to examine whether G-Re promotes the proliferation of MSCs through its estrogen-like activity. The cultures were treated with G-Re (10 $\mu\text{mol/L}$) or estradiol (E₂) in the presence and absence of ICI 182, 780 (an estrogen receptor antagonist), and with ICI 182, 780 alone or vehicle alone, namely, being divided into six groups referred to as Re, Re + ICI, E₂, E₂ + ICI, ICI, and VC, respectively. Figure 3 shows the obtained results. These two values of both Re and E₂ groups were significantly higher than those of the VC group. Similarly, the cell counts and MTT readings (per well) of expanded MSCs at passage 3 in Re and E₂ groups were significantly greater than those of VC groups.

Meanwhile, not only the areas ($3.10 \pm 0.63 \text{ mm}^2$ per well) and yield (22.37 ± 2.55 per 10^6 NBMCs) of CFU-F colonies but also the counts [$(5.91 \pm 0.33) \times 10^3$ per well] and MTT readings (0.76 ± 0.07) of expanded MSCs in E₂ + ICI groups were markedly reduced compared with E₂ groups, but also these measurements obtained from Re + ICI groups were significantly lower than those from Re groups. In addition, no significant difference of these four measurements was observed between ICI and any one of VC, Re + ICI, and E₂ + ICI groups; meanwhile, there were significant differences between the ICI group and Re or E₂ group (statistical analysis results of differences between these groups in the following experiments are consistent with what is described here, and the same results will not be repeated hereinafter). These findings suggested that estrogen receptor–mediated signaling was involved in proliferation-promoting effects of G-Re on the MSCs.

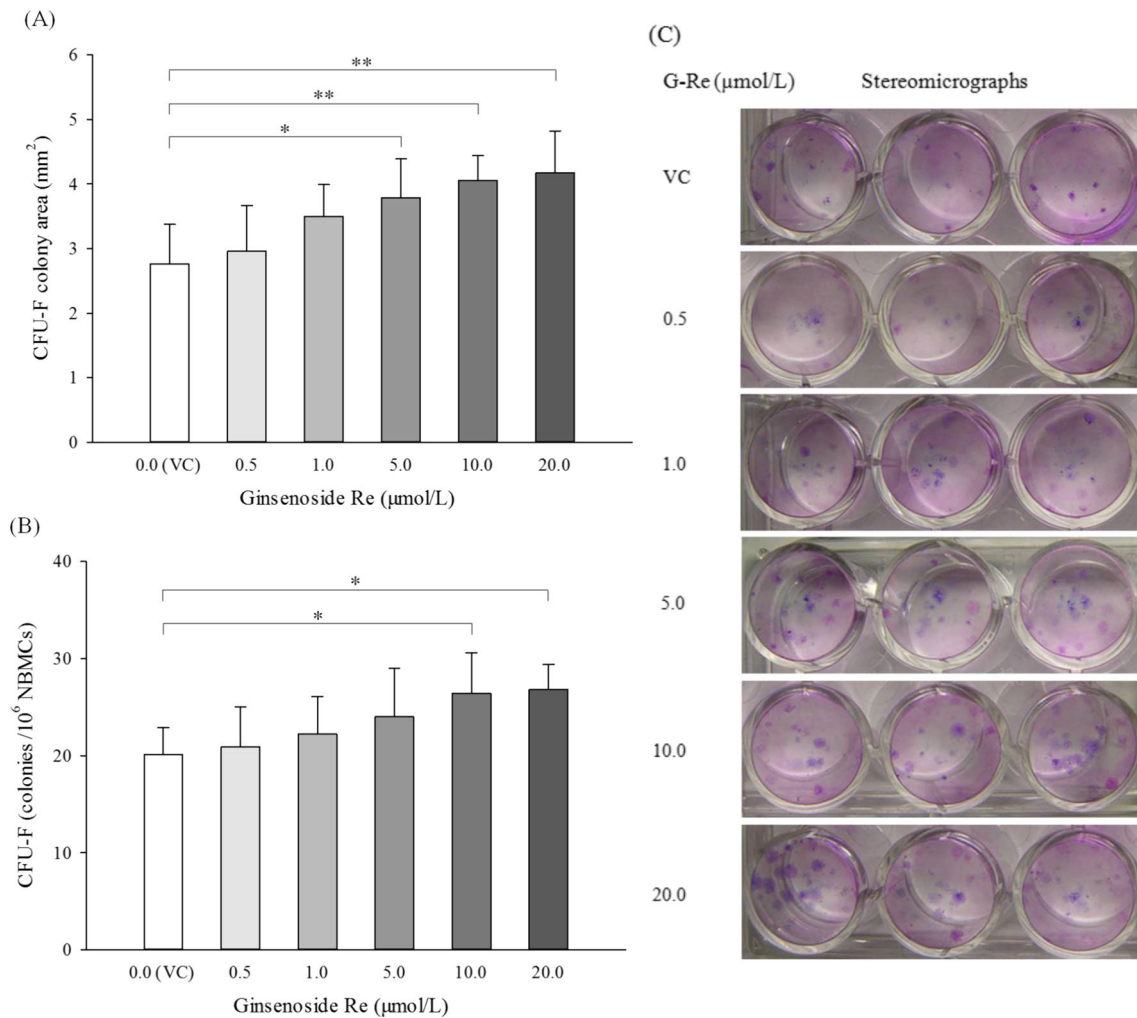


Figure 1. The proliferation-promoting effect of ginsenoside Re on CFU-Fs. (A) The colonies of CFU-F were developed in the 9-d cultures of freshly isolated bone marrow cells which were seeded in 24-well plates at various concentrations of G-Re. The area values of CFU-F colonies were calculated from the 10 largest CFU-F colonies per well in triplicate for every experiment. (B) The yields of CFU-F colonies were calculated as the number of counted CFU-F colonies per 10^6 plated NBMCs. In both panels A and B, each column with

a bar represents mean \pm SD of four independent experiments. * and ** denote $P < 0.05$ and $P < 0.01$, respectively, to indicate significant differences as compared with the vehicle-control group (one-way ANOVA followed by the Fisher's least square difference test, $n = 4$). (C) The stereomicrographs of CFU-F colonies grown in culture wells were taken from a representative experiment. Acronyms and abbreviations: NBMCs, nucleated bone marrow cells; CFU-F, colony-forming unit fibroblast; VC, vehicle control; G-Re, ginsenoside Re.

GS-Re exerts estrogen-like effect on cell cycle distribution of expanded MSCs To confirm the estrogen-like effect of GS-Re on the proliferation, MSCs were mediated by inducing changes in cell cycle progression. The effect of G-Re on cell cycle phase distribution was assessed in the culture-expanded MSCs. The cells were divided into the six groups: Re, Re + ICI, E_2 , E_2 + ICI, ICI, and VC as described in the preceding section. The proportion of cells in G_0/G_1 , S, and G_2/M phases was determined by flow cytometry and the results are shown in Fig. 3. In comparison with the VC group, the percentages of S + G_2/M phase cells were markedly increased in both Re and E_2 groups, concomitant with significant decreases in the percentages of G_0/G_1 phase cells.

The percentages of cells in S + G_2/M phases were respectively 13.51% in the VC group, 24.12% in the Re group, and 27.09% in the E_2 group, and of course, the proportions of cells in G_0/G_1 phases were the results of a hundred percent minus the percentages of S + G_2/M phase cells in the corresponding groups. The addition of estrogen antagonist ICI 182, 780 to the culture media was able to markedly reduce the cell cycle effect of either G-Re or estradiol on the expanded MSCs. The percentage of the cell populations in the S + G_2/M phases in Re + ICI and E_2 + ICI groups was significantly lower than those in the Re group (15.66% vs. 24.12%) and the E_2 group (12.69% vs. 27.09%), respectively. Thus, the flow cytometric analysis provided evidences that

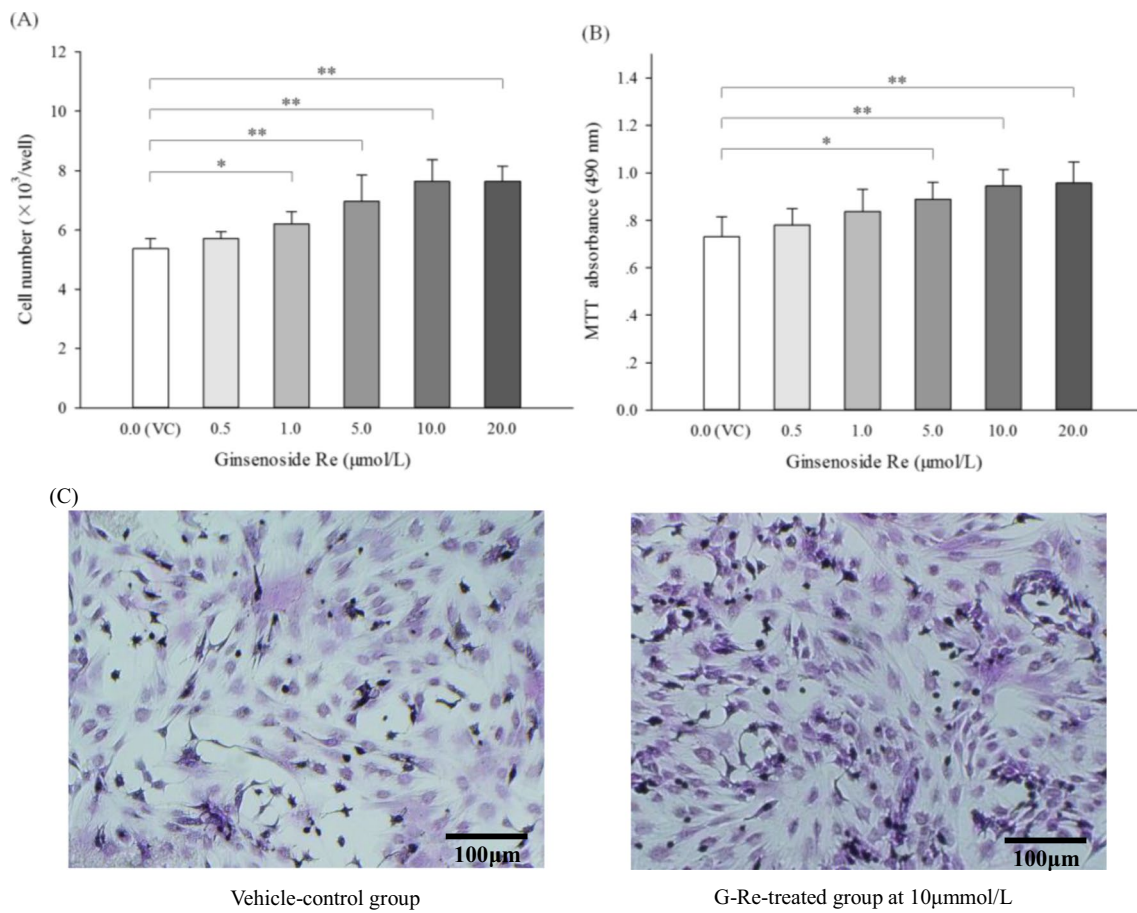


Figure 2. The proliferation-promoting effect of ginsenoside Re on culture-expanded MSCs. (A) The cell number was determined after the third passage MSCs had been cultured in 96-well plates at the indicated concentrations of G-Re for 2 d. The number of cells per well was calculated according to counted cells in 10 randomly selected microscopic fields in triplicate wells for every experiment. (B) The optical absorbance of each well was measured by MTT assays for evaluating the cell viability and proliferative activity of the third passage MSCs exposed to G-Re at the indicated concentrations. In both panels A and B, each column with a bar represents

mean \pm SD of four independent experiments. * and ** denote $P < 0.05$ and $P < 0.01$, respectively, to indicate significant differences as compared with control (one-way ANOVA followed by the Fisher's least square difference test, $n = 4$). (C) Two representative microphotographs of the third passage MSCs were taken from vehicle control and 10 $\mu\text{mol/L}$ G-Re-treated groups, respectively (Wright-Giemsa staining and original magnification $\times 100$). The scale bar = 100 μm . Acronyms and abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; and see the legend of Fig. 1 for others.

the estrogen-like effect of GS-Re on the proliferation of MSCs could be mediated through alteration of cell cycle progression.

Ginsenoside Re stimulates DNA synthesis of expanded MSCs through its estrogen-like activity To further evaluate the mechanisms underlying estrogen-like action of G-Re on MSC proliferation, immunofluorescence staining was employed to measure the percentage of bromodeoxyuridine (BrdU)-positive cells into which BrdU is incorporated during DNA synthesis at the S phase of the cell cycle. MSCs at passage 3 were serum-starved to synchronize the cell cycle at G0/G1 phases, and then were divided into the six groups: Re, Re + ICI, ICI, E₂, E₂ + ICI, and VC as stated above. After an incubation period of 24 h, all of the cell populations

were exposed to BrdU overnight. The percentage of BrdU-positive nuclei in each group was obtained. The results are shown in Fig. 4. The percentages of BrdU-positive cells in both Re (35.27%) and E₂ (39.30%) groups were significantly higher than that of the VC group (25.20%). The addition of ICI 182, 780 to the culture media virtually abrogated either G-Re- or estradiol-stimulated effects on BrdU incorporation in the expanded MSCs, and the percentages of BrdU-positive cells were decreased to 23.43% in the Re + ICI group and to 28.20% in the E₂ + ICI group, respectively. Moreover, no significant difference in the percentages of BrdU-incorporated cells was observed between ICI and VC groups. These results indicate that G-Re is able to enhance DNA synthesis through an estrogen receptor-dependent mechanism.

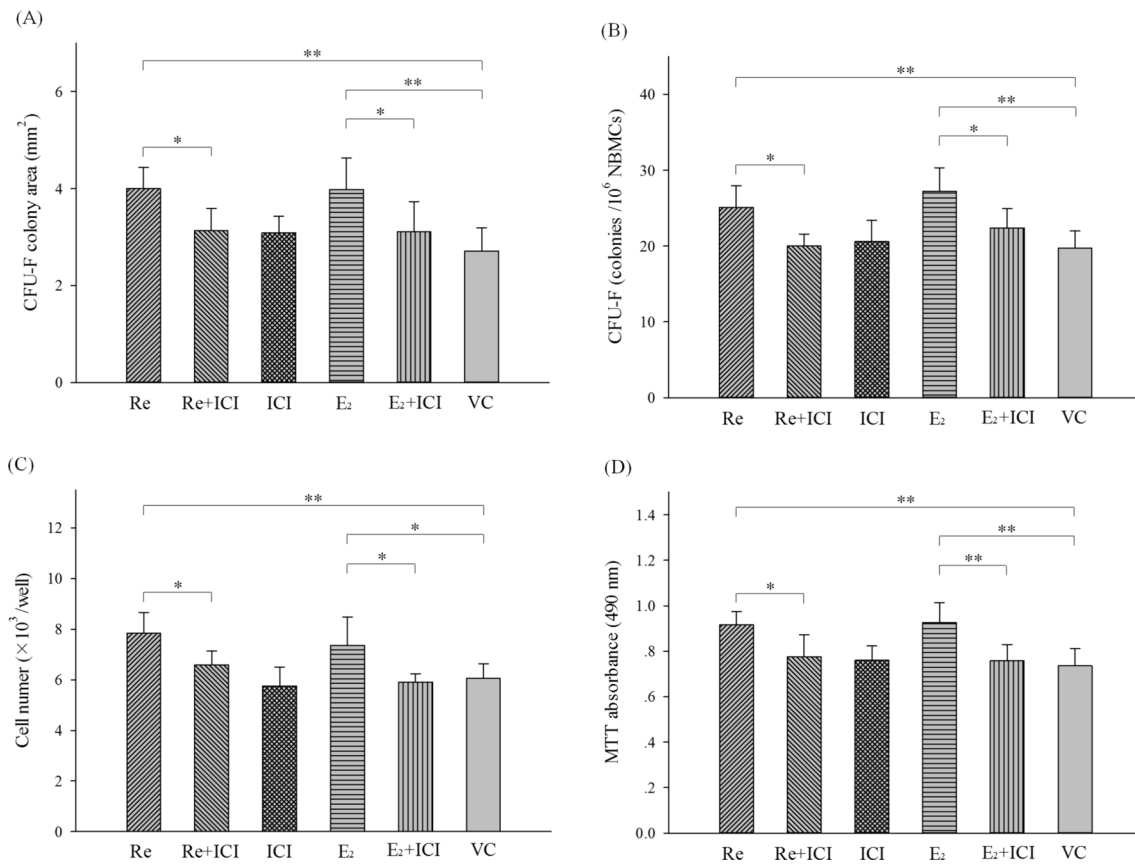


Figure 3. The estrogen-like effects of ginsenoside Re on the proliferation of primary and expanded MSCs. The freshly isolated bone marrow cells and the third passage MSCs were incubated for 48 and 24 h, respectively, and non-adherent cells were removed. The adherent cells were treated with G-Re (10 $\mu\text{mol/L}$) or estradiol in the presence and absence ICI 182, 780 (an estrogen receptor antagonist), or with ICI 182, 780 alone, or with vehicle (growth medium containing 0.05% (v/v) ethanol) alone, that is to say, both of them were divided into six groups: Re, Re+ICI, E₂, E₂+ICI, ICI, and VC. The cells were incubated for the indicated time periods. (A) The area values of CFU-F colonies were obtained from the 10 largest CFU-F colonies per well in triplicate for every experiment. (B) The yields of

CFU-F colonies were calculated as the number of counted CFU-F colonies per 10⁶ plated NBMCs. (C) The number of cells per well was calculated according to the third passage MSCs in 10 randomly selected microscopic fields in triplicate wells for every experiment. (D) The MTT absorbance at 490 nm was measured from the treated MSCs at passage 3. For in all panels, each column with a bar represents mean \pm SD of four independent experiments. * and ** denote $P < 0.05$ and $P < 0.01$, respectively, to express significant differences between pairing groups as indicated by the lines below asterisks (one-way ANOVA followed by Fisher's least square difference test, $n = 4$). Acronyms and abbreviations: E₂, estradiol; ICI, ICI 182, 780; and see the legend of Figs. 1 and 2 for others.

MAPK/ERK signaling is involved in estrogen-like effects of ginsenoside Re on the expanded MSCs Western blot immunoassay was used to determine the expression level of p-ERK1/2, so as to assess whether the ERK1/2-related signaling pathway contributes to the proliferation-promoting effect of GS-Re on the MSCs. The third passage MSCs were divided into Re, Re + ICI, ICI, E₂, E₂ + ICI, and VC groups. These cells were cultured for 24 h, and then their p-ERK1/2 levels were measured. The results are shown in Fig. 5. In comparison with the VC group, the expression levels of p-ERK1/2 in both GS-Re and estradiol groups were increased, while ICI182780 could stop from upregulation of GS-Re and E₂ on expression of p-ERK1/2. In terms of the fold change over the VC group, the expression levels of p-ERK1/2 in Re, Re+ICI, ICI, E₂, and E₂+ICI groups

were 1.47 ± 0.24 , 1.14 ± 0.06 , 0.89 ± 0.19 , 1.51 ± 0.17 , and 0.93 ± 0.37 , respectively. The expression level of p-ERK1/2 in Re or E₂ groups was significantly higher than that in the VC group, and also higher than that in the ICI group. No obvious difference in the expression level of p-ERK1/2 was observed between ICI and VC groups (Fig. 6). These results indicate that the activation of ERK1/2 contribute to the positive effect of GS-Re on the proliferation of MSCs.

Discussion

In the current study, G-Re was found to enhance the in vitro proliferation of MSCs derived from the murine bone marrow. In the first place, this assertion has been evidenced by

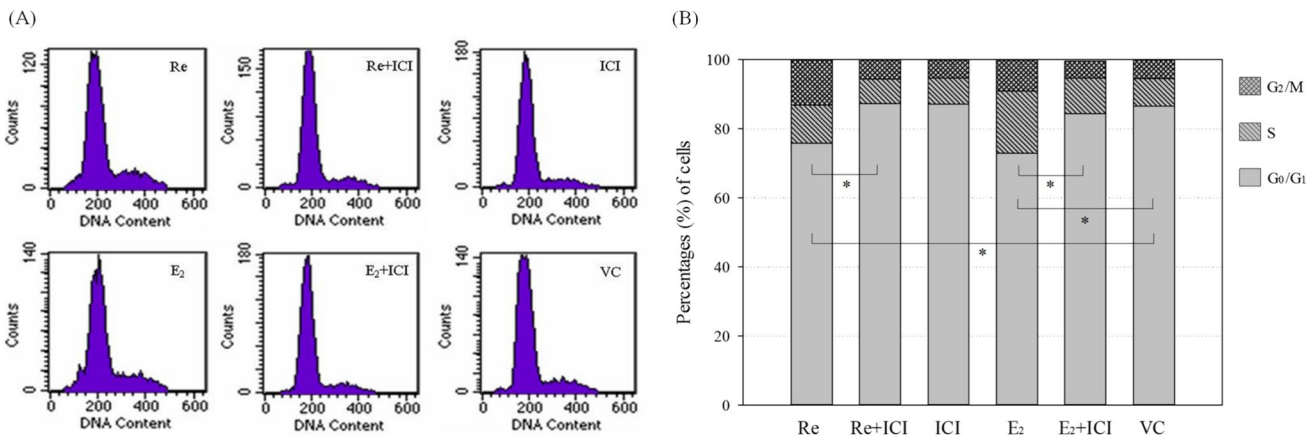


Figure 4. Flow cytometric analysis of the estrogen-like effect of ginsenoside Re on cell cycle distribution in expanded MSCs. MSCs at passage 3 were incubated for 24 h and non-adherent cells were discarded. The adherent cells were divided into six groups: Re, Re + ICI, ICI, E₂, E₂ + ICI, and VC as mentioned above. After another 24 h of culture, the cells were harvested for flow cytometric analysis. For each cell population, DNA contents of at least 10,000 cells were measured based on propidium iodide staining. Quantitative analysis of cell cycle phase distribution was performed using ModFit LT soft-

ware. (A) The histograms representative of three independent experiments is shown. (B) The percentages of cells in G₀/G₁, S, and G₂/M phases of each group are the pooled data from three separate experiments. The asterisks (*) express statistical significance of differences between pairing groups as indicated by the lines above asterisks (chi-square test for the R×C contingency tables, with the partitioned chi-square method for multiple comparisons; the overall significance level=0.05, and the Bonferroni-adjusted significance level=0.003). Acronyms and abbreviations: see the legend of Fig. 2 for others.

CFU-F colony forming assay of the primary bone marrow cells. The results showed that the average area and frequency of CFU-F colonies were increased following the exposure of primary bone marrow cells to G-Re. The CFU-F assay is considered as a gold standard method to characterize MSCs (Penforinis and Pochampally 2016). The size of CFU-F colony can represent the proliferative rate of MSCs since a colony is formed as a consequence of the clonal expansion of a MSC after a period of in vitro culture. Also, the frequency of CFU-F colonies from the cultured cells can be used as an indicator for the proliferative activity of MSCs, seeing that a cellular aggregation will not be counted as a colony unless the fibroblast-like cells constituting it reach a certain number. The size and frequency of CFU-F colony have been frequently used by many laboratories to evaluate the proliferative activity of MSCs (Gronthos and Simmons 1995; Berger *et al.* 2020). Furthermore, the cell counting and MTT assay were performed to evaluate the effects of G-Re on the proliferation of culture-expanded MSCs. After the third-passage MSCs were exposed to G-Re at different concentrations, the expanded cells were scored directly using a counting method for monolayer cells attached to culture plate in situ. The elevated cell counts in groups treated with G-Re indicate that this ginsenoside monomer is able to promote the proliferation of expanded MSCs. Increases in MTT absorbance values of the expanded MSCs exposed to different concentrations of G-Re further demonstrate the proliferation-promoting effect of G-Re on these cells. Some researchers have documented that G-Rg1 enhanced the in vitro proliferation of human, rat, and mouse MSCs

derived from the bone marrow and adipose tissue (Lu *et al.* 2008; Xiao *et al.* 2022). Most recently, Ling presented the evidences that G-Rg1 increased the proliferative activity of human amnion-derived MSCs (Ling *et al.* 2023). Hong and coworkers reported the positive effect of G-Rg3 on the proliferation of human bone marrow MSCs (Hong *et al.* 2020).

The promoting effect of G-Re on the in vitro proliferation of MSCs derived from the murine bone marrow exhibited a dose-dependency. The progressive increase in proliferative response of MSCs to the rise G-Re concentration was observed in the range of 0.5 to 20 μmol/L. A couple of scientific teams reported previously that ginsenoside Rg1 enhanced the expansion of human and rat MSCs at different concentrations in vitro, with the greatest effects at the 1.2, 5.0, 10.0, and 40.0 μmol/L, respectively (Wang *et al.* 2014; Gu *et al.* 2016; Wang *et al.* 2020). Hong and colleagues described that MSCs derived from human bone marrow displayed the increased proliferation when these cells were exposed to ginsenoside Rg3, the maximal stimulation of which was attained at 60 μmol/L. G-Re has been shown to exert its actions on the in vitro proliferation of other cell types in a dose-dependent manner. In a study on human umbilical vein endothelial cell, the cell proliferation was significantly enhanced in the presence of G-Re in the culture and reached a maximal level at a concentration of 30 μg/mL (≈ 31.7 μmol/L) (Huang *et al.* 2005). Li observed that G-Re promoted the cyclic growth of murine hair follicles in 48-h and 72-h culture durations at 10 mg/L (≈ 10.6 μmol/L), but not at 50 mg/L (≈ 52.8 μmol/L) (Li *et al.* 2016). Our findings have indicated that the optimal concentration of G-Re for the

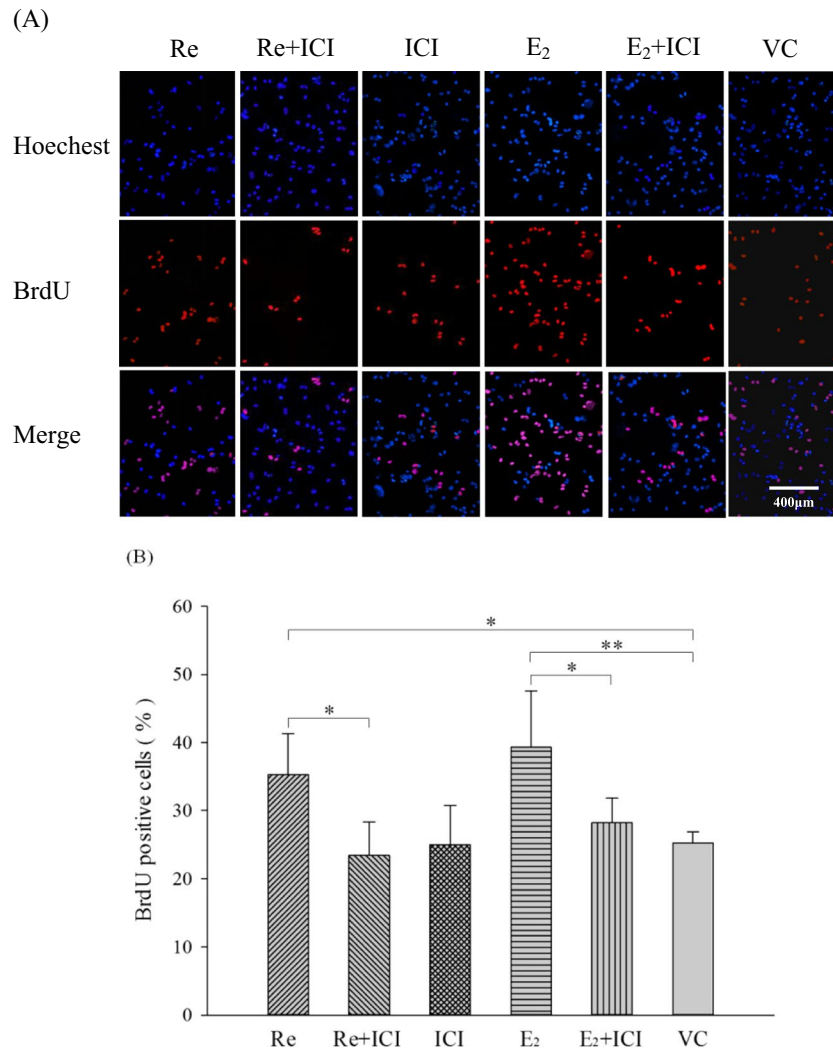


Figure 5. Immunofluorescence analysis of BrdU incorporation for the estrogen-like effect of ginsenoside Re on the expanded MSCs. The third passage MSCs were grown to reach sub-confluent state, and then serum-starved to synchronize the cell cycle at G₀/G₁ phases. After serum-free media were replaced by normal media, the cells were divided into the aforesaid six groups: Re, Re+ICI, ICI, E₂, E₂+ICI, and VC and successively incubated for 24 h. BrdU was added to medium in every group and the cells were incubated overnight. BrdU-positive nuclei was detected by immunofluorescence staining. (A) The fluorescence photomicrographs shown are representative of three separate experiments. Total nuclei are indicated

by blue staining, and the BrdU incorporated nuclei are indicated by red staining. A scale bar of 400 μ m is shared among all microphotographs in this panel. Original magnification $\times 100$. (B) 1000 nuclei were microscopically counted from at least 10 random fields per cover slip. Each column represents a percentage of the pooled data from each group over three independent experiments. * and ** denote $P < 0.05$ and $P < 0.01$, respectively, to express significant differences between pairing groups as indicated by the lines below asterisks (one-way ANOVA followed by Fisher's least square difference test, $n = 3$). Acronyms and abbreviations: Hoechst, Hoechst 33,342; BrdU, bromodeoxyuridine; and see the legend of Fig. 2 for others.

enhancement of MSC proliferation in vitro is 10 μ mol/L, which will be used for the subsequent experiments.

Estrogen-like activities of ginsenosides have been described in the literature. Several researcher groups reported that G-Re can produce its effects through the cell signaling pathways involved in steroid hormones. Bae *et al.* stated that G-Re exhibited a weak estrogenic activity in human breast carcinoma MCF-7 cells (Bae *et al.* 2005). Furukawa and coworkers provided evidences that G-Re activates endothelial NO synthase (eNOS) to

release NO, resulting in activation of the cardiac potassium channel, via a signaling pathway of each of androgen receptor, estrogen receptor- α , and progesterone receptor (Furukawa *et al.* 2006). A result obtained by Nakaya *et al.* from the experimental study of vascular smooth muscle cells showed that G-Re-induced activation of eNOS and large-conductance Ca²⁺-activated potassium channel was blocked by ICI 182, 780, an inhibitor of estrogen receptors, suggesting that eNOS activation occurred via a signaling pathway associated with estrogen receptors as well. In the

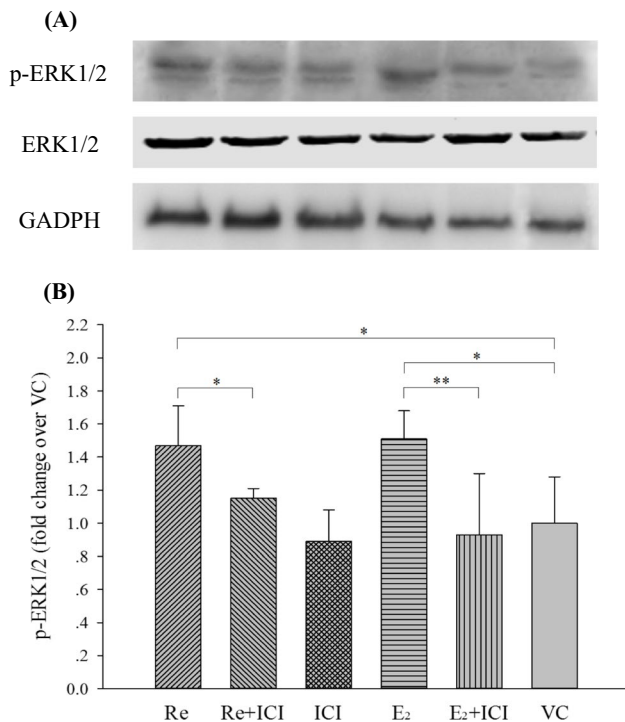


Figure 6. Quantitative analysis of phosphorylated ERK1/2 protein in the expanded MSCs exposed to ginsenoside Re. After the third passage, MSCs were incubated for 24 h; non-adherent cells were discarded. The adherent cells were divided into the aforesaid six groups: Re, Re+ICI, ICI, E₂, E₂+ICI, and VC. Another 24 h later, the expression levels of p-ERK1/2 were measured by Western blotting. (A) A representative result of Western blot immunoassay is shown. (B) Each column with a bar represents mean \pm SD of three independent experiments. * and ** denote $P < 0.05$ and $P < 0.01$, respectively, to express significant differences between pairing groups as indicated by the lines below asterisks (one-way ANOVA followed by Fisher's least square difference test, $n = 3$). Acronyms and abbreviations: GADPH, glyceraldehyde-3-phosphate dehydrogenase; p-ERK1/2, phosphorylated extracellular signal-regulated kinases 1 and 2; and see the legend of Fig. 2 for others.

study presented herein, we have assessed the estrogen-like effects of G-Re on the proliferation of MSCs using estradiol as a positive control and ICI 182,780 to block the activation of estrogen receptors. The obtained results prove that proliferation-promoting effect of G-Re on MSCs is estrogen receptor-dependent. It has been demonstrated that MSCs express all three subtypes of estrogen receptor (ER), namely ER α , ER β , and GPER (Zhang *et al.* 2016; Chuang *et al.* 2020). The promoting action of estradiol on the proliferation of MSCs in dose- and time-dependent manners has been described in a number of papers, and is mainly mediated by ER α (Hong *et al.* 2011). Whether ginsenosides exert their effects on the proliferation of MSCs through the activation of ERs, as far as we know, was documented only by Lu *et al.* They reported that the proliferation of culture-expanded bone marrow stromal cells was

enhanced by exposure of G-Rg1, and the activation of ER α was essential for mediating this effect of G-Rg1. However, G-Rg1 might activate ER α in a ligand-independent manner for the reason that it failed to interact directly with the estrogen receptor as indicated by ER binding assay in their study (Lu *et al.* 2008). Before that, Chan and colleagues had found that G-Rg1 was unable to displace the specific binding of estradiol to MCF-7 cell lysate (Chan *et al.* 2002). A few years later, estrogen-responsive element (ERE)-based luciferase reporter experiment of Laua *et al.* showed that G-Rg1 preferentially activates ER α via the phosphorylation of AF-1 domain in the absence of receptor binding (Lau *et al.* 2008). It should be noted that confusing and contradictory results for binding assays of G-Re to the estrogen receptor were presented by two groups of researchers, respectively. In the study of Furukawa *et al.* competitive binding assays suggested that G-Re could bind to ER α , and the fluorescence resonance energy transfer experiment manifested that G-Re competitively inhibited estradiol-induced coactivator recruitment to ligand binding domain (LBD) of ER α (Furukawa *et al.* 2006). In contrast, Lee reported that G-Re did not bind to estrogen receptors as examined by the transcription activation of the luciferase reporter genes in transiently transfected CV-1 cells with the corresponding steroid hormone receptors and reporter plasmids containing hormone responsive element (Lee *et al.* 2003). Our study has demonstrated that the promoting effect of G-Re on proliferation of MSCs depends heavily on the involvement of estrogen receptor, and further experiments are needed to clarify the exact signaling mechanisms underlying its actions.

Numerous studies have indicated that ginsenosides alter the cell proliferation rate through the modulation of cell cycle progression in various types of cells (Hu *et al.* 2015; He and Yao 2021). In order to confirm that the proliferation-promoting effect of G-Re on MSC is mediated through the acceleration of cell cycle progression, we utilized the flow cytometry and the BrdU immunofluorescence staining to analyze the cell cycle phase distribution. It was shown, by the flow cytometric analysis, that the percentages of S + G₂/M phase cells were significantly increased in both Re and E₂ groups accompanied by a significant decrease in the percentages of G₀/G₁ phase cells as compared to the VC group. The cell cycle effects of either G-Re or estradiol on MSCs were almost reversed by concomitant treatment with estrogen antagonist ICI-182780. These measurements suggest that G-Re is able to promote the transition of MSCs from G₀/G₁ to S phase, resulting in the accumulation of dividing cells at the S phase of the cell cycle. The promotion of G₁-to-S phase shift generally results in a given population of cells to contain a larger fraction of S phase cells which can be measured by staining for incorporated BrdU. Our experimental results indicated that the frequency

of BrdU-labeled nuclei was increased in cell (MSC) population exposed to G-Re or estradiol, and effects of the two treatment agents were also reversed by ICI-182780. Consequently, the analyses of flow cytometry and BrdU staining data demonstrate that G-Re exerts the mitogenic action on MSCs via accelerating cell cycle progression.

Multiple signaling pathways participate in the regulation of MSC proliferation. Among them, the extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway plays a central role in diverse cellular processes. ERK1/2 is activated by phosphorylation and conveys growth and mitogenic signals to a wide variety of cellular components to control cell proliferation. A number of studies have demonstrated that mitogenic effects of many extracellular cues, such as growth factors, estrogens, phytoestrogens, and hypoxia, on MSCs are mediated through activation of ERK1/2 (Schilling *et al.* 2014; Yun *et al.* 2009; Lavoie *et al.* 2020). As a first choice, therefore, it was tested whether promoting effect of G-Re on the MSC proliferation depends on the activation of ERK1/2 signaling pathway by Western blot immunoassay in this study. Our findings indicated that the treatment of G-Re upregulated the expression level of phosphorylated ERK1/2 in the expanded MSCs, and estradiol exhibited a consistent positive effect. ICI 182, 780 could antagonize the action of either G-Re or estradiol. The results here suggest that the activation of the ERK1/2 signaling pathway is involved in the proliferative response of MSCs to GS-Re. To our knowledge, there has not been any research published yet about ERK1/2 activation contributing to the promotion of ginsenosides on MSC proliferation. Several groups of authors have reported that G-Re affects biological behaviors of other cell types due to the involvement of the ERK1/2 pathway. Wang and coworkers documented that the facilitation of G-Re on Schwann cell proliferation depended on the phosphorylation of ERK1/2 and JNK1/2. In an investigation on neuronal differentiation of Neuro-2a cells, Kim *et al.* observed the activation of ERK by G-Re. Qiao *et al.* examined the neuroprotective effect of G-Re against neurotoxin-induced Parkinson's disease using the human neuroblastoma cell line SH-SY5Y as an *in vitro* model, and showed the enhancing effect of G-Re on the phosphorylation of ERK. However, Li reported that G-Re suppressed TGF- β -induced phosphorylation of ERK in HeLa cells, and Gao *et al.* stated that G-Re attenuated LPS-induced Ras/ERK1/2 expression in vascular smooth muscle cells *in vivo* and *in vitro* (Gao *et al.* 2019). Such seemingly contradictory observations could result from different factors including cell type, cell context, and other factors. Certainly, the intrinsic cellular signaling mechanisms of how G-Re enhances the proliferation of MSCs remain to be addressed.

Our present research has mainly focused on the estrogen-like effects of G-Re on the proliferation of MSCs taking into account the biological and clinical relevance of

maintaining their proliferative ability. Many studies have provided evidences that the proliferative capacity of MSCs is reduced with aging, even though certain authors failed to observe such a phenomenon. The decline in the proliferative activity of MSCs has also occurred in certain diseases and disorders, including osteoporosis, Fanconi anemia, and obesity (Frazier *et al.* 2013; Li *et al.* 2014; Zhou *et al.* 2017). Estrogen replacement therapy (ERT) has been widely used in naturally menopausal women for the prevention and treatment of osteoporosis, cardiovascular events, and other health problems, and in women with early menopause or premature ovarian insufficiency as well. Long-term ERT, however, has reported to increase the risk of breast, ovarian, and endometrial cancers (Armeni *et al.* 2021). Some researchers have been seeking for suitable substitutes for ERT which are expected to avoid the risk of gynecological cancers and other adverse side effects. Ginseng is considered to be one of the promising substitutes for ERT. It should be noted that ginseng contains many bioactive constituents, including various ginsenosides which are recognized as the main bioactive components. Ginsenosides can exhibit diverse biological activities and may produce either positive synergistic or undesirable antagonistic effects in the body. The pharmacological counteraction between ginsenosides possibly reduces the resulting efficacy of ginseng (Lee *et al.* 2022; Li *et al.* 2022). Thus, it is worth attempting to clarify the effect of ginsenoside monomer, especially major bioactive saponins, so as to develop effective ginsenoside-based therapeutic agents and dietary supplements. Previous studies have been shown that G-Re could exert estrogen-like effects on several cell types but did not promote growth of the cultured breast cancer cell line MCF-7 (Bae *et al.* 2005; Lu *et al.* 2008). Herein, the experimental results reported by us will provide the useful information for the further researches on G-Re.

In summary, the present study demonstrates that ginsenoside Re exerts a promoting effect on the *in vitro* proliferation of MSCs derived from murine bone marrow in a concentration-dependent manner. The estrogen receptor-mediated signaling pathway is involved in this effect. These findings will be helpful for further studies intended to elucidate the more precise mechanisms underlying the action of ginsenoside Re on the MSC proliferation.

Author contribution Linzi Luo performed major experimental work and paper drafting. Bin Peng and Lei Xiong performed partial experimental work and collected the data. Baohe Wang performed paper revision and design instruction. Linghao Wang designed the study and supported funding resource. All authors read and approved the final manuscript.

Funding This work was supported by the Natural Science Foundation of Hunan Province (CN) (No. 2023JJ30846).

Data availability The data used to support the findings of this study are available from the corresponding author upon request.

Declarations

Conflict of interest The authors declare no competing interests.

References

- Armeni E, Paschou SA, Goulis DG, Lambrinouadaki I (2021) Hormone therapy regimens for managing the menopause and premature ovarian insufficiency. *Best Pract Res Clin Endocrinol Metab* 35:101561
- Bae EA, Shin JE, Kim DH (2005) Metabolism of ginsenoside Re by human intestinal microflora and its estrogenic effect. *Biol Pharm Bull* 28:1903–1908
- Berger DR, Aune ET, Centeno CJ, Steinmetz NJ (2020) Cryopreserved bone marrow aspirate concentrate as a cell source for the colony-forming unit fibroblast assay. *Cytherapy* 22:486–493
- Bhat S, Viswanathan P, Chandanala S (2021) Expansion and characterization of bone marrow derived human mesenchymal stromal cells in serum-free conditions. *Sci Rep* 11:3403
- Bilia AR, Bergonzi MC (2020) The G115 standardized ginseng extract: an example for safety, efficacy, and quality of an herbal medicine. *J Ginseng Res* 44:179–193
- Chan RY, Chen WF, Dong A, Guo D, Wong MS (2002) Estrogen-like activity of ginsenoside Rg1 derived from *Panax notoginseng*. *J Clin Endocrinol Metab* 87:3691–3695
- Christensen LP (2009) Ginsenosides chemistry, biosynthesis, analysis, and potential health effects. *Adv Food Nutr Res* 55:1–99
- Chuang SC, Chen CH, Chou YS (2020) G protein-coupled estrogen receptor mediates cell proliferation through the cAMP/PKA/CREB pathway in murine bone marrow mesenchymal stem cells. *Int J Mol Sci* 21:6490
- Deng H, Sun C, Sun Y (2018) Lipid, protein, and microRNA composition within mesenchymal stem cell-derived exosomes. *Cell Reprogram* 20:178–186
- Ferrin I, Beloqui I, Zabaleta L (2017) Isolation, culture, and expansion of mesenchymal stem cells. *Methods Mol Biol* 1590:177–190
- Frazier TP, Gimble JM, Devay JW, Tucker HA, Chiu ES, Rowan BG (2013) Body mass index affects proliferation and osteogenic differentiation of human subcutaneous adipose tissue-derived stem cells. *BMC Cell Biol* 7(14):34
- Friedenstein AJ, Chailakhjan RK, Lalykina KS (1970) The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* 3:393–403
- Furukawa T, Bai CX, Kaihara A, Ozaki E, Kawano T, Nakaya Y, Awais M, Sato M, Umezawa Y, Kurokawa J (2006) Ginsenoside Re, a main phytoesterol of *Panax ginseng*, activates cardiac potassium channels via a nongenomic pathway of sex hormones. *Mol Pharmacol* 70:1916–1924
- Gao XY, Liu GC, Zhang JX (2022) Pharmacological properties of ginsenoside Re. *Front Pharmacol* 13:754191
- Gao Y, Zhu P, Xu SF (2019) Ginsenoside Re inhibits PDGF-BB-induced VSMC proliferation via the eNOS/NO/cGMP pathway. *Biomed Pharmacother* 115:108934
- Gronthos S, Simmons PJ (1995) The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions in vitro. *Blood* 85:929–940
- Gu Y, Zhou J, Wang Q, Fan W, Yin G (2016) Ginsenoside Rg1 promotes osteogenic differentiation of rBMSCs and healing of rat tibial fractures through regulation of GR-dependent BMP-2/SMAD signaling. *Sci Rep* 6:25282
- Hade MD, Suire CN, Suo Z (2021) Mesenchymal stem cell-derived exosomes: applications in regenerative medicine. *Cells* 10:1959
- Haynesworth SE, Baber MA, Caplan AI (1996) Cytokine expression by human marrow-derived mesenchymal progenitor cells in vitro: effects of dexamethasone and IL-1 alpha. *J Cell Physiol* 166:585–592
- He F, Yao G (2021) Ginsenoside Rg1 as a potential regulator of hematopoietic stem/progenitor cells. *Stem Cells Int* 2021:4633270
- Hong L, Zhang G, Sultana H, Yu Y, Wei Z (2011) The effects of 17- β estradiol on enhancing proliferation of human bone marrow mesenchymal stromal cells in vitro. *Stem Cells Dev* 20:925–931
- Hong T, Kim MY, Da Ly D (2020) Ca(2+)-activated mitochondrial biogenesis and functions improve stem cell fate in Rg3-treated human mesenchymal stem cells. *Stem Cell Res Ther* 11:467
- Hu W, Jing Wang P (2015) The positive effects of ginsenoside Rg1 upon the hematopoietic microenvironment in a D-galactose-induced aged rat model. *BMC Complement Altern Med* 15:119
- Huang YC, Chen CT, Chen SC, Lai PH, Liang HC, Chang Y, Yu LC, Sung HW (2005) A natural compound (ginsenoside Re) isolated from *Panax ginseng* as a novel angiogenic agent for tissue regeneration. *Pharm Res* 22:636–646
- Kim YK, Yoo DS, Xu H (2009) Ginsenoside content of berries and roots of three typical Korean ginseng (*Panax ginseng*) cultivars. *Nat Prod Commun* 4:903–906
- Lau WS, Chan RY, Guo DA, Wong MS (2008) Ginsenoside Rg1 exerts estrogen-like activities via ligand-independent activation of ER α pathway. *J Steroid Biochem Mol Biol* 108:64–71
- Lavoie H, Gagnon J, Therrien M (2020) ERK signalling: a master regulator of cell behaviour, life and fate. *Nat Rev Mol Cell Biol* 21:607–632
- Lee HW, Ang L, Lee MS (2022) Using ginseng for menopausal women's health care: a systematic review of randomized placebo-controlled trials. *Complement Ther Clin Pract* 48:101615
- Lee YJ, Jin YR, Lim WC, Ji SM, Cho JY, Ban JJ, Lee SK (2003) Ginsenoside Rc and Re stimulate c-fos expression in MCF-7 human breast carcinoma cells. *Arch Pharm Res* 26:53–57
- Levy M, Crawford JR, Dib N (2019) Phase I/II study of safety and preliminary efficacy of intravenous allogeneic mesenchymal stem cells in chronic stroke. *Stroke* 50:2835–2841
- Li Y, Charif N, Mainard D (2014) Donor's age dependent proliferation decrease of human bone marrow mesenchymal stem cells is linked to diminished clonogenicity. *Biomed Mater Eng* 24:47–52
- Li Y, Yan MY, Chen QC (2022) Current research on complementary and alternative medicine in the treatment of premature ovarian failure: an update review. *Evid Based Complement Alternat Med* 2022:2574438
- Li Z, Ryu SW, Lee J, Choi K, Kim S, Choi C (2016) Protopanaxatrol type ginsenoside Re promotes cyclic growth of hair follicles via inhibiting transforming growth factor β signaling cascades. *Biochem Biophys Res Commun* 470:924–929
- Lim KH, Lim DJ, Kim JH (2013) Ginsenoside-Re ameliorates ischemia and reperfusion injury in the heart: a hemodynamics approach. *J Ginseng Res* 37:283–292
- Ling L, Shu H, Huang Y, Hou J, Hua Y (2023) Effects of ginsenoside Rg1 on the biological behavior of human amnion-derived mesenchymal stem/stromal cells (hAD-MSCs). *Stem Cells Int* 2023:7074703
- Liu M, Lei H, Dong P (2017) Adipose-derived mesenchymal stem cells from the elderly exhibit decreased migration and differentiation abilities with senescent properties. *Cell Transplant* 26:1505–1519
- Liu Y, Liu N, Li X, Luo Z, Zhang J (2022) Ginsenoside Rb1 modulates the migration of bone-derived mesenchymal stem cells through the SDF-1/CXCR4 axis and PI3K/Akt pathway. *Dis Markers* 2022:5196682

- Lu XZ, Wang JH, Wu X, Zhou L, Wang L, Zhang XW, Cao KJ, Huang J (2008) Ginsenoside Rg1 promotes bone marrow stromal cells proliferation via the activation of the estrogen receptor-mediated signaling pathway. *Acta Pharmacol Sin* 29:1209–1214
- Mohanan P, Subramaniam S, Mathiyalagan R, Yang DC (2018) Molecular signaling of ginsenosides Rb1, Rg1, and Rg3 and their mode of actions. *J Ginseng Res* 42:123–132
- Perfornis P, Pochampally R (2016) Colony forming unit assays. *Methods Mol Biol* 1416:159–169
- Piao X, Zhang H, Kang JP (2020) Advances in saponin diversity of Panax ginseng. *Molecules* 25:3452
- Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* 284:143–147
- Schilling T, Ebert R, Raaijmakers N, Schütze N, Jakob F (2014) Effects of phytoestrogens and other plant-derived compounds on mesenchymal stem cells, bone maintenance and regeneration. *J Steroid Biochem Mol Biol* 139:252–261
- Shi ZY, Zeng JZ, Wong AST (2019) Chemical structures and pharmacological profiles of ginseng saponins. *Molecules* 24:2443
- Siegel G, Kluba T, Hermanutz-Klein U, Bieback K, Northoff H, Schäfer R (2013) Phenotype, donor age and gender affect function of human bone marrow-derived mesenchymal stromal cells. *BMC Med* 11:146
- Sun Y, Li W, Lu Z, Chen R, Ling J, Ran Q, Jilka RL, Chen XD (2011) Rescuing replication and osteogenesis of aged mesenchymal stem cells by exposure to a young extracellular matrix. *FASEB J* 25:1474–1485
- Wagner W, Horn P, Castoldi M, Diehlmann A, Bork S, Saffrich R, Benes V, Blake J, Pfister S, Eckstein V, Ho AD (2008) Replicative senescence of mesenchymal stem cells: a continuous and organized process. *PLoS ONE* 3:e2213
- Wang P, Wei X, Zhang F (2014) Ginsenoside Rg1 of Panax ginseng stimulates the proliferation, odontogenic/osteogenic differentiation and gene expression profiles of human dental pulp stem cells. *Phytomedicine* 21:177–183
- Wang Z, Jiang R, Wang L (2020) Ginsenoside Rg1 improves differentiation by inhibiting senescence of human bone marrow mesenchymal stem cell via GSK-3 β and β -catenin. *Stem Cells Int* 2020:2365814
- Xiao L, Wang M, Zou K, Li Z, Luo J (2022) Effects of ginsenoside Rg1 on proliferation and directed differentiation of human umbilical cord mesenchymal stem cells into neural stem cells. *NeuroReport* 33:413–421
- Yosupov N, Haimov H, Juodzbaly G (2017) Mobilization, isolation and characterization of stem cells from peripheral blood: a systematic review. *J Oral Maxillofac Res* 31(8):e1
- Yun SP, Lee MY, Ryu JM, Song CH, Han HJ (2009) Role of HIF-1 α and VEGF in human mesenchymal stem cell proliferation by 17 β -estradiol: involvement of PKC, PI3K/Akt, and MAPKs. *Am J Physiol Cell Physiol* 296:C317–C326
- Zanichelli F, Capasso S, Cipollaro M, Pagnotta E, Carteni M, Casale F, Iori R, Galderisi U (2012) Dose-dependent effects of R-sulforaphane isothiocyanate on the biology of human mesenchymal stem cells, at dietary amounts, it promotes cell proliferation and reduces senescence and apoptosis, while at anti-cancer drug doses, it has a cytotoxic effect. *Age (Dordr)* 34:281–293
- Zhang F, Tang S, Zhao L (2021) Stem-leaves of Panax as a rich and sustainable source of less-polar ginsenosides: comparison of ginsenosides from Panax ginseng, American ginseng and Panax notoginseng prepared by heating and acid treatment. *J Ginseng Res* 45:163–175
- Zhang W, Schull S, Du M, Liu J, Lu Z, Zhu H, Xue S, Lian F (2016) Estrogen receptor α and β in mouse: adipose-derived stem cell proliferation, migration, and brown adipogenesis in vitro. *Cell Physiol Biochem* 38:2285–2299
- Zhou Y, He Y, Xing W (2017) An abnormal bone marrow microenvironment contributes to hematopoietic dysfunction in Fanconi anemia. *Haematologica* 102:1017–1027

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.