Protective effects of ginsenoside Rg1 on aging Sca-1$^+$ hematopoietic cells

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Abstract. In adults, bone hematopoietic cells are responsible for the lifelong production of all blood cells. It is affected in aging, with progressive loss of physiological integrity leading to impaired function by cellular intrinsic and extrinsic factors. However, intervention measures, which directly inhibit the aging of hematopoietic cells, remain to be investigated. In the present study, 10 μmol/l ginsenoside Rg1 (Rg1) markedly alleviated the aging phenotypes of Sca-1$^+$ hematopoietic cells following *in vitro* exposure. In addition, the protective effects of ginsenoside Rg1 on the aging of Sca-1$^+$ hematopoietic cells was confirmed using a serial transplantation assay in C57BL/6 mice. The mechanistic investigations revealed that Rg1-mediated Sca-1$^+$ hematopoietic cell aging alleviation was linked to a series of characteristic events, including telomere end attrition compensation, telomerase activity reconstitution and the activation of genes involved in *p16-Rb* signaling pathways. Based on the above results, it was concluded that ginsenoside Rg1 is a potent agent, which acts on hematopoietic cells to protect them from aging, which has implications for therapeutic approaches in hemopoietic diseases.

Introduction

The foundations of maintenance of the adult circulatory system are based on hematopoietic cells, particularly hematopoietic stem cells (HSCs), which are responsible for the lifelong production of all blood lineages. Accumulating evidence indicates that the number and functional properties of hematopoietic cells in aged animals are altered to become the opposite of those in younger counterparts (1). The loss of function of bone hematopoietic cells leads to myeloid skewing of differentiation at the expense of lymphopoiesis (2,3). Disruption of the functions of normal bone hematopoietic cell functions is responsible for diverse leukemia subtypes and immune deficiencies due to aging and other physiological and pathological stimuli (4).

It well known that HSCs express c-kit and Sca-1 (Ly-6 A/E) cell surface molecules and lack lineage markers (Sca-1$^+$ Lin$^-$c-kit$^+$ cells). Sca-1 is a glycosylphosphatidylinositol-linked cell surface protein found on hematopoietic stem cells in the mouse (5). Sca-1 has been reported to be necessary for normal HSC activity and is involved in determining the fate of hematopoietic progenitor/stem cells, as Sca-1-knockout mice have defects in short-term competitive transplantation and serial transplantation (6,7). Further evidence has revealed that the proliferative response of Thy-1^{low} Sca-1$^+$Lin$^-$c-kit$^+$ cells from aged mice to the FMS-like tyrosine kinase 3 ligand and thrombopoietin is markedly decreased (8,9). It may be considered that Sca-1$^+$ cells, to a certain extent, represent a population cells involved in hematopoiesis and blood homeostasis.

Panax ginseng is used in traditional Chinese medicine to enhance stamina and overcome physical stress over past decades. The beneficial effects of ginseng and its constituents in terms of its anticancer and immunomodulatory effects have also been reported (10). It has been demonstrated that ginsenoside, one of >25 constituents derived from ginseng, has various functions, including anti-aging, antioxidant and immunomodulatory effects (11,12). Ginsenoside Rg1(Rg1), one of the neutral saponins of Ginseng root, is purified using its crystalline decaacetate (13). The structure of this saponin has been established as 6,20-di-O-β-glucosyl-20*S*-protopanax atriol (14). It has been demonstrated that Rg1 exerts protective effects on human endothelial cells and PC12 cells (15,16). Rg1 also increases neural plasticity in efficacy and structure, and the proliferation and differentiation of neural progenitor cells in the dentate gyrus of the hippocampus of normal adult mice and a global ischemia model in gerbils (17). It has also been reported that Rg1 in *P. ginseng* increases CD4$^+$ T-cell activity and the proportion of T helper cells among the total number of T cells, and promotes the gene expression of IL-2 in murine splenocytes (13,18).

Based on this information, the present study hypothesized that Rg1 has a protective effect on aged hematopoietic cells.

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The present study investigated the effects of Rg1 on Sca1+ hematopoietic cells using *in vitro* exposure experiments. As a positive control, tert-butyl hydroperoxide (t-BHP) was selected, owing to pervious study demonstrating that 100 μmol/l t-BHP effectively induced Sca-1+ cell senescence following 6 h co-culture *in vitro* (19). Replicative senescent Sca1+ cells were from serial transplanted C57BL/6 mice were also used to investigate the effects of Rg1 on the aging process of Sca1+ cell subpopulation *in vivo*. The present study also examined changes in the chromosome end telomere system of Sca1+ cells and other senescence-associated biomarkers. The aim of these investigations was to determine the effects of Rg1 in the maintenance of hematopoietic cell function and tissue homeostasis.

Materials and methods

The study was approved by the Ethics Committee of Chongqing Medical University (Chongqing, China). Rg1 (purity, 96% of *P. ginseng*) was purchased from Jinlin Hongjiu Co., Ltd. (Changchun, China), Iscove's modified Dulbecco's medium (IMDM) culture medium was provided by Gibco Life Technologies (Carslbad, CA, USA), fetal bovine serum (FBS) and horse serum were purchased from Sijiqing Co, Ltd. (Hangzhou, China), an Anti-Sca-1+ Micro Bead kit was obtained from Miltenyi Biotec GmbH (Berhisch Gladbach, Germany), t-BHP and Ficoll separation solution were obtained from Sigma-Aldrich (St. Louis, MO, USA), methylcellulose was obtained from (Stemcell Technologies, Inc., Vancouver, Canada), telomere probes were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA), a genomic DNA extraction kit, DNA Ladder and Hinf I endonucleases were obtained from Sangon Biotech, Co., Ltd. (Shanghai, China), the telomere repeat amplification protocol-quantitative polymerase chain reaction (TRAP-qPCR) silver stain telomerase activity detection kit was obtained from KeyGEN Co., Ltd. (Shanghai, China), an ECL kit was obtained from Pierce Biotechnology (Rockford, IL, USA). Rabbit anti-CDKN2A/p16-INK4a (cat. no. bs-0740R), rabbit anti-CDK2 (cat. no. bs-10726R), rabbit anti-phospho-Rb (Ser780; cat. no. bs-1347R), rabbit anti-CDK4 (cat. no. bs-0633R), goat anti-Mouse IgG/RBITC (cat. no. bs-0296G-RBITC) and horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1:500) antibodies were obtained from Bioss (Beijing, China), and were diluted with sterile phosphate-buffered saline (PBS). The western blotting kit and the SA-β-Gal staining kit was from Beyotime Institute of Biotechnology (Shanghai, China).

Animals and cell culture. C57BL/6 mice (6-8 weeks; 20-25 g) were bred in-house in a pathogen-free environment at a temperature of 22-25˚C. The Institutional Animal Care and Use Committee of Chongqing Medical University (Chongquing, China) approved all animal experiments, which were performed according to Guide for the Care and Use of Laboratory Animals (20). The bone marrow suspension, obtained from the femur and tibia of the C57BL/6 mice with a needle was subjected to Ficoll-Paque density gradient separation to isolate bone mononuclear cells, followed by Sca-1+ cell separation using magnetic-activated cell sorting (MACS). The Sca-1+ cells were cultured in IMDM supplemented with 10% FBS at 37˚C in a humidified atmosphere containing 5% CO_2 prior to drug exposure. Each group contained 5 mice unless otherwise specified.

Serial transplantation assay. Briefly, the Sca-1+ cells were isolated and purified from male C57BL/6 mice bone marrow mono-nucleated cells using MACS and cultured in IMDM supplemented with 10% fetal bovine serum. A total of 10,000 harvested Sca-1+ cells from male C57BL/6 mice were injected into the lateral tail veins of lethally irradiated (8.5 Gy $Co^{60}\gamma$) female C57BL/6 mice. Mice were irradiated to 8.5 Gy in an Cammacell-40 Exposure Instrument (Atomic Energy of Canada Limited, Gloucester, Canada) at a dose-rate of 1.0 Gy per minute for 8.5 min. At 4 weeks post-transplantation, the recipients were used as donors for a subsequent transplantation cycle and for *in vitro* assays. Serial transplants were performed in both male and female C57BL/6 mice strains. Engraftment efficiency in the C57BL/6 mice recipients was monitored by detecting the Sry gene of the Y-chromosome using PCR for donor contribution (35 cycles of 94˚C for 4 min, 94˚C for 20 sec, 60˚C for 30 sec and 72˚C for 30 sec; primer sequences (Bio-Rad, Hercules, CA, USA), forw ard 5'-GAAAAGCCTTACAGAAGCCGA-3' and reverse 5'-G TATGTGATGGCATGTGGGTTC-3'; 211 base pairs). Serial transplantation assays were performed three times in independent experiments, with five recipient mice/group/experiment. The animals were monitored daily for the presence of disease, and were sacrificed by decapitation under diethyl ether (Tianhao Chemical Industry, Guangdong, China) anesthetics at designated times-points following transplantation, or when moribund. The experiments were performed following dividing of the 20 adult female C57BL/6 mice into the four following groups, each containing five mice: Group 1, mice were irradiated with total 8.5 Gy $Co^{60}\gamma$ and injected with 0.2 ml PBS via the lateral tail vein, designated the control); group 2, mice were irradiated with total 8.5 Gy $Co^{60}\gamma$ followed by intraperitoneal administration with 20 mg/kg/d Rg1 for 4 weeks as the positive control (designated Rg1); group 3, mice were irradiated with total 8.5 Gy $Co^{60}\gamma$ followed by injection with 10,000 Sca-1+ cells via tail vein transplantation, designated the negative control (T) group; group 4, mice were administered intraperitoneally with 20 mg/kg/d Rg1 for 1 month following Sca-1+ cell transplantation, designated T+Rg1. The Sca-1+ cells isolated from each group of mice were then assessed for aging biomarkers.

Senescence-associated β-galactosidase (SA-β-Gal) activity assay. The cells (10,000) were fixed with 0.5% glutaraldehyde for 15 min, followed by washing twice with PBS and incubation with 1 ml SA-β-Gal staining solution at 37˚C without CO_2 for 24 h. The number of positively stained cells were stochastically counted within 100 cells using a charge-coupled device camera attached to a phase-contrast microscope (CKX41-A32RC; Olympus, Tokyo, Japan). All counting was performed in triplicate.

Colony formation assay. The cells from all the groups were respectively harvested and plated ($3x10^3$/well) in a 96-well plate, with each well containing 1.1 ml 0.8% methylcellulose (Sigma-Aldrich) in an environment of 37˚C, 5% CO_2

for 2 weeks prior to fixation with 4% paraformaldehyde and staining with 0.1% crystal violet. The colony numbers were counted under an optical microscope (CX22; Olympus, Tokyo, Japan). A cell sphere containing >50 cells was considered one colony.

Western blot analysis. For western blot analysis, cellular proteins were obtained from the total cell lysates resuspended in double distilled H_2O. The cellular proteins (30 μg) were electrophoresed using SDS-poly-acrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (GE Healthcare Life Sciences, Chalfont, UK). The membrane was blocked with 5% skim milk in PBS for 2 h at room temperature and then incubated with specific antibodies, anti-P16, anti-Rb, anti-CDK2, anti-CDK4 and anti-cyclin E (Bioss) all at 1:200 dilutions at 4˚C overnight. HRP-conjugated goat anti-rabbit antibodies were used as a secondary antibody, at 1:5,000 dilution for 2 h at room temperature. The specific proteins were detected using enhanced chemiluminescent reagents (Pierce Biotechnology). Densitometry quantification was performed using the Image-Pro Plus v 6.0 software (Media Cybernetics, Inc., Bethesda, MD, USA).

Southern blot analysis. Genomic DNA from the six treatment groups were digested with Hinf/RsaI enzyme at 37˚C for 2 h. Electrophoresis of the digested genomic DNA (20 μg) was performed in 0.7% agarose gels (Yeasen, Shanghai, China) at 1 V/cm. Following electrophoresis, the gels were denatured and neutralized; and the DNA was transferred in 20X SSC onto nylon membrane filters. The membrane was prehybridization at 42˚C for 1 h in 10 ml prehybridization solution, containing 3 m 20X SSC, 1 ml 50X Denhardt solution, 0.5 ml 10% SDS, and 5.5 ml H_2O (Klamar, Shanghai, China), followed by incubated at 42˚C overnight following the addition of telomere probe (100 ng/ml) . Finally, the membrane was washed twice in 2X SSC containing 0.1% SDS (Klamar) for 30 min, blocked for 15 min, and mixed by agitation with streptavidin-HRP (1:400; Boster, Wuhan, China) buffer at 37˚C for 40 min. The membrane was then washed in PBS three times, followed by 10 min incubation with 3,3'-diaminobenzidine and capture of images. The intensity of the signals were determined using AlphaView System software (Alpha Innotech Corporation, St. San Leandro, CA, USA) and the mean lengths of the TRFs were calculated with $L=\Sigma (ODi:l:Li)=\Sigma (ODi)$.

Target region amplified polymorphism (TRAP)-quantitative (q)PCR assay. A total of $1x10^6$ cells were washed once in ice-cold wash buffer, resuspended and centrifuged at 800 x g for 5 min at 4˚C. The precipitation was homogenized with 40 μl cold lysis buffer and lysad for 30 min in ice, followed by centrifugation at 1,500 rpm for 30 min at 4˚C and collection of the supernatant (optical density 260/280 of RNA: 1.8-2.0). To the extension reaction, 50 μl ofa solution, containing 5 μl 10X TRAP buffer, 1 μl dNTPs, 1 μl Taq-DNA polymerase, 1 μl TS primer, 2 μl telomerase extraction, 39 μl DEPC H_2O and 1 μl CX primer was added. Prior to qPCR, solution without CX primer was pre-incubated at 23˚C for 30 min. The PCR for the TRAP assay was performed using the following program: 94˚C for 5 min; 35 cycles at 94˚C for 30 sec, 50˚C for 30 sec, 72˚C for 90 sec; 72˚C for 10 min. SYBR Green dye (1 μl) was added to the PCR tube, mixed well, and incubated for 10 min at room temperature. PCR was conducted using a Bio-Rad sequence detection system (cfx96; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The fluorescence intensity was determined using a fluorospectrophotometer (Bio-Rad Laboratories, Inc.), and the ratio of telomerase activity/fluorescence intensity:protein concentration was determined.

Data analysis. All values are expressed as the mean ± standard deviation. Analyses were performed using repeated measures of analysis of variance and an unpaired two-tailed Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Ginsenoside Rg1 has a protective effect on normal and aged Sca-1$^+$ cells following in vitro exposure. To observe the effects of Rg1 on Sca-1$^+$ cells *in vitro*, the Sca-1$^+$ cells were treated with an achievable plasma concentration of 10 μmol/l Rg1. To enable direct comparison with the effect of Rg1 on aging cells, side by side investigations were performed using t-BHP, owing to the fact that 100 μmol/l t-BHP effectively induces Sca-1$^+$ cells aging following 6 h *in vitro* co-culture (19). The results of the SA-β-Gal staining assay demonstrated that there was a marked increase following the cells treated with 100 μmol/l t-BHP, compared with the control group cells. Otherwise, no significantly difference in the number of positively-stained Sca-1$^+$ were observed cells between the groups treated with or without 10 μmol/l Rg1. To determine whether Rg1 had protective effects on the aging Sca-1$^+$ cells, the Sca-1$^+$ cells were primarily cultured with 100 μmol/l t-BHP for 6 h, and then washed with sterile PBS and transferred into 10 μmol/l Rg1 medium for another 12 h co-culture. Following the SA-β-Gal staining assay, the numbers of positively-stained Sca-1$^+$ cells were counted. As shown in Fig. 1, the higher rate of positive staining of the Sca-1$^+$ cells caused by t-BHP exposure decreased following treatment with Rg1 (8.33±2.41, 7.04±2.53, 68.50±4.95 and 30.08±2.44 for the control, Rg1, t-BHP and Rg1+t-BHP groups, respectively).

To determine whether Rg1 functionally affected the ability of Sca-1$^+$ cells to form colonies, methylcellulose colony-forming unit (CFU) assays were performed. The results revealed that Sca-1$^+$ cells exposed to 10 μmol/l Rg1 exhibited a minor/moderate increase in CFUs. By contrast, the Sca-1$^+$ cells exposed to 100 μmol/l t-BHP exhibited a significant decrease in CFUs. The decreased abilities of Sca-1$^+$ cells to form colonies caused by t-BHP was apparently compensated for by treatment with 10 μmol/l Rg1 (15.04±1.78, 18.25±2.27, 3.34±1.58 and 9.34±2.23 CFUs in the control, Rg1, t-BHP and Rg1+t-BHP groups, respectively; Fig. 1). These data indicated that Rg1 had a protective effect on normal and on t-BHP-induced aged Sca-1$^+$ cells *in vitro*.

Rg1-mediated Sca-1$^+$cell senescence alleviation is dependent on the regulation of p16 and downstream cell cycle regulators. It is known that the *$p16^{INK4a}$/pRb* signaling pathway is important in the process of replicative senescence in fibroblasts. To gain insight into the molecular basis of

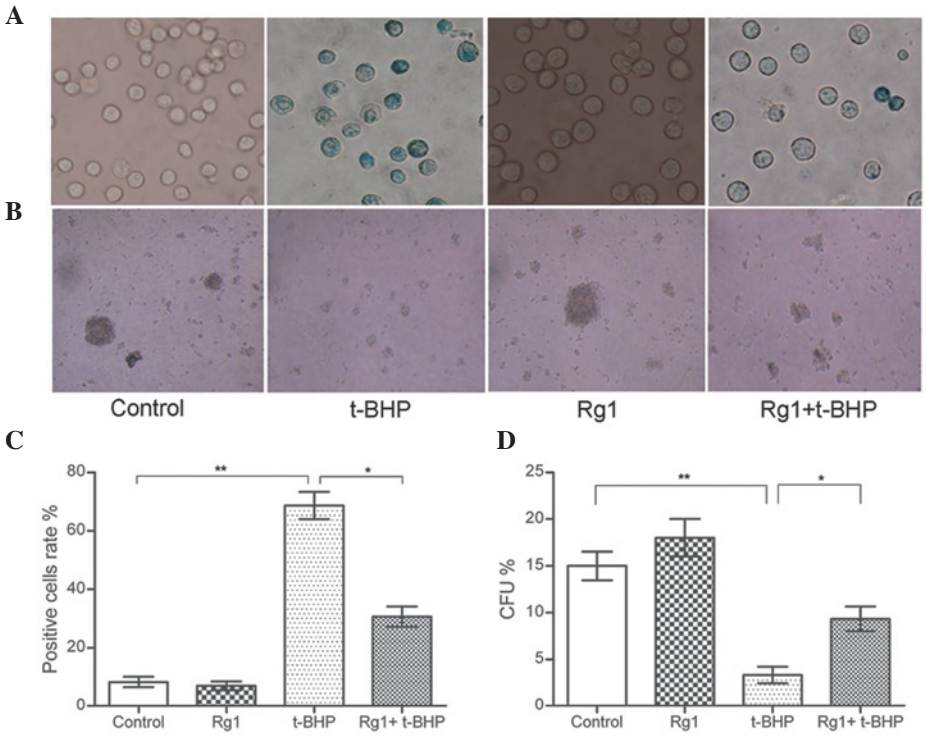

Figure 1. Rg1 has a protective effect on normal and aged Sca-1+ cells *in vitro*. (A) Images of positive Sca-1+ cells expressing β-galactosidase, visualized under an inverted microscope. Magnification, x400. (B) Images of CFUs formed by Sca-1+ cells under an inverted microscope. Magnification, x200. (C) Histogram representation of the quantitative percentage of positively-stained Sca-1+ cells in different treatment groups (n=6/group) (D) Histogram representation of the quantitative percentage of CFUs in different treatment groups. (n=5/group) Data are expressed as as the mean ± standard deviation. *P<0.05; **P<0.01. Rg1, ginsenoside Rg1; t-BHP, tert-butyl hydroperoxide; control, untreated cells; CFU, colony forming unit.

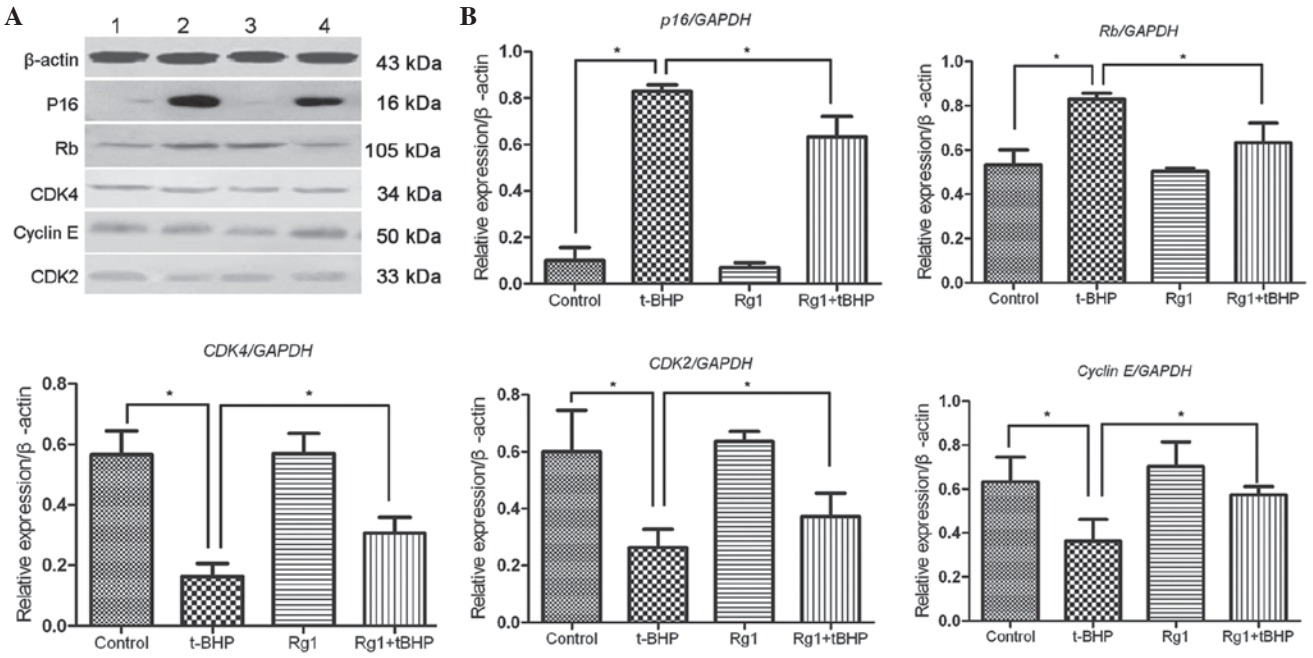

Figure 2. Rg1-mediated alleviation of Sca-1+ cell senescence is dependent on the regulation of p16 and downstream cell cycle regulators. (A) Protein expression levels of P16, Rb, CDK4, Cyclin E and CDK2 of the Sca-1+ cells in different treatment groups. 1, control; 2, t-BHP; 3, Rg1; 4, t-BHP+Rg1. (B) Quantification of respective proteins expression levels in Sca-1+ cells in different groups is represented as histograms. Data are expressed as the mean ± standard deviation (n=3). *P<0.05. Rg1, ginsenoside Rg1; t-BHP, tert-butyl hydroperoxide; control, untreated cells.

Rg1-mediated senescence alleviation in Sca-1+ cells, western blot analysis was performed to examine the expression patterns of the important senescence-asssociated proteins, P16 and Rb, in Sca-1+ cells exposed to different agents. The results demonstrated that the protein expression levels of P16 and Rb are markedly increased following t-BHP exposure

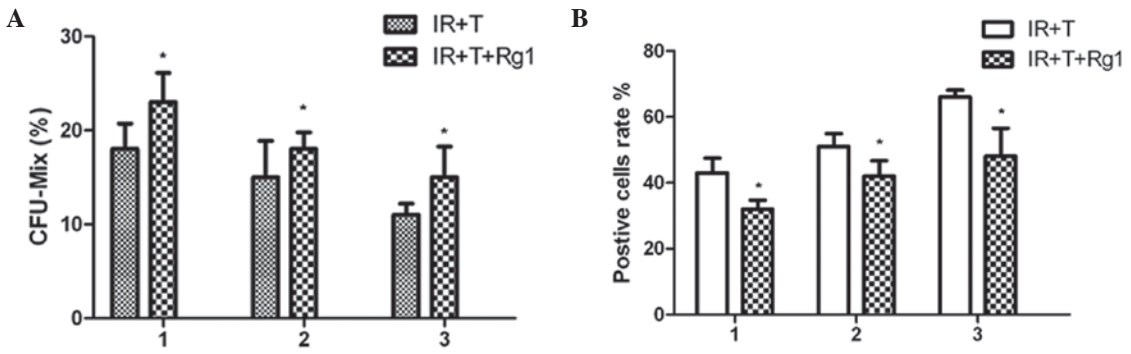

Figure 3. Rg1 restores the aging profile of Sca-1$^+$ cells in mice. (A) Histogram representation of the quantitative percentage of positively stained Sca-1$^+$ cells expressing β-galactosidase in the IR+T and IR+T+Rg1 groups of cells following three rounds of transplantation (n=3) (B) Histogram representation of the quantitative percentage of CFUs in the IR+T (irradiation and then transplantation) and IR+T+Rg1 (irradiation and then transplantation following Rg1 administration) groups of cells following three rounds of transplantation (n=5). Data are expressed as the mean ± standard deviation. *P<0.05, vs. IR+T. Rg1, ginsenoside Rg1; IR+T, irradiation and then transplantation; IR+T+Rg1, irradiation and transplantation following Rg1 administration; CFU, colony forming unit.

in vitro. However, the higher protein expression levels of P16 and Rb in t-BHP-mediated Sca-1$^+$ cells was decreased by 10 μmol/l Rg1 *in vitro*. Cell senescence is linked with specific changes in the expression of cell cycle regulators, contributing to cell proliferation arrest. To confirm the protective effects of Rg1, the present study further analyzed the expression levels of CDK2, CDK4 and cyclin E in the Sca-1$^+$ cells. Rg1 was observed to significantly restore the higher protein expression levels of CDK2, CDK4 and cyclin E in the t-BHP-induced Sca-1$^+$ cells. No other detectable differences were observed in the levels of protein expression between the Sca-1$^+$ cells with or without Rg1 exposure *in vitro* (Fig. 2). These results implied that Rg1 substantially alleviated the cell cycle arrest, which was induced by t-BHP *in vitro*, and was associated with the regulation of *p16^{INK4a}/pRb* signaling.

Ginsenoside Rg1 restores the aging profile of Sca-1$^+$ cells in mice. To validate the protective effects of Rg1 on Sca-1$^+$ hematopoietic cells in mice, a serial transplantation assaywas performed and Sca-1$^+$ cells were collected from male mice and transplanted into lethally irradiated female mice to reconstitute the hematopoietic compartment. At 4 weeks post-transplantatation, the functional changes of the Sca-1$^+$ cells were assessed using a colony forming units assay. These results indicated that the Rg1-treated Sca-1$^+$ cells had higher colony-forming capacity, leading to an increased number of CFUs, compared with the control group of untreated cells (Fig. 3). The first indication of increased colony-forming capacity in the Rg1-treated Sca-1$^+$ cells transplanted into the primary recipient mice was evident from the evaluated level of colony-forming capacity in Sca-1$^+$ cells obtained from secondary recipients. At 8 weeks post-transplantation, the Rg1-treated Sca-1$^+$ cells from secondary recipient mice exhibited higher colony-forming capacity compared with the cells in the control group. A similar tendency was also observed in the colony-forming capacity of the Sca-1$^+$ cells from the third recipient mice.

To determine the aging profile of the Sca-1$^+$ cells during the process of transplantation, BM Sca-1$^+$ cells from the Rg1-treated or control group recipient mice were analyzed using an SA-β-Gal staining assay. The results revealed an increased number of positively stained cells within the transplant process, indicating that the regulation of replicative senescence was, at least in part, responsible for Sca-1$^+$ cell aging during the process of serial transplantation. The beneficial effects of Rg1 on Sca-1$^+$ cells aging was evidenced by the return of the aging cells close to normal levels following Rg1 treatment.

p16-Rb signaling is responsible for Rg1-mediated Sca-1$^+$ cells aging alleviation in mice. To gain insight into the basis for Rg1 on Sca-1$^+$ cells in mice, Sca-1$^+$ cells from the tibial and femoral bone marrow of the third recipient mice were collected and analyzed using western blotting to observe the age-associated changes in the protein expression levels of *p16-Rb* signaling pathways. The results demonstrated that there were decreases in the levels of P16 and Rb, but increases in the levels of CDK2, CDK4 and cyclin E in the Rg1-treated mice, compared with the control group (Fig. 4). These findings correlated with a significant change in the expression levels of P16, Rb, CDK2, CDK4 and cyclin E in the Sca-1$^+$ cells *in vitro* assays. These results implied that *p16^{INK4a}/pRb* signaling was responsible for the regulation of Rg1 on Sca-1$^+$ cells aging alleviation in mice.

Rg1 functions as telomere elongation and telomerase maintenance of aged Sca-1$^+$ cells in mice. Telomeres are formed by tandem repeats of the TTAGGG sequence, which has a base attrition loss during each cell division. Telomere shortening progressively occurs during cell replicative senescence. Abnormalities in the telomere length and telomerase activities of the recipient mice further promoted an examination of the senescence-associated *p16-Rb* signaling pathways (21). To analyze whether Rg1-mediated Sca-1$^+$ cells aging alleviation is linked to telomere changes, the present study performed southern blotting to observe changes in telomere length in Sca-1$^+$ cells from the third recipient mice. As shown in Fig. 5, the telomere lengths of the Sca-1$^+$ cells from recipient mice were marginally decreased, compared with the control group cells, whereas a marked restoration in the telomere length of the Sca-1$^+$ cells was identified following Rg1 treatment. This recovery was observed at similar time-points in three independent experiments. Telomeres are important in the aging of

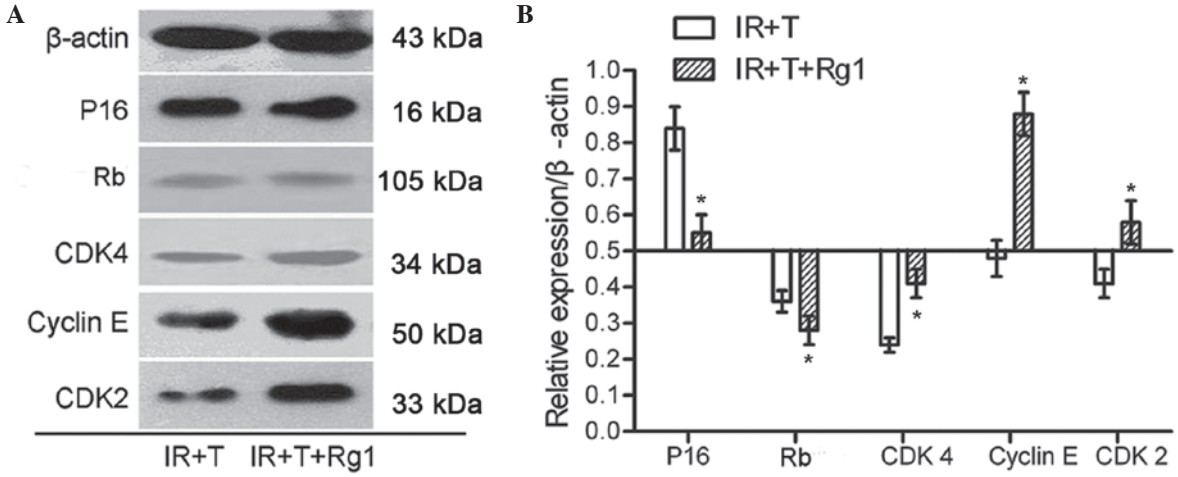

Figure 4. *p16-Rb* signaling is responsible for Rg1-mediated alleviation of Sca-1+ cells in mice. (A) Protein expression levels of P16, Rb, CDK4, Cyclin E and CDK2 of the Sca-1+ cells in IR+T and IR+T+Rg1 groups of cells from the third transplantation recipient mice. (B) Quantification of the respective protein expression levels of Sca-1+ cells in the different groups. Data are expressed as the mean ± standard deviation (n=3) *P<0.05, IR+T+Rg1 vs. IR+T. Rg1, ginsenoside Rg1; IR+T, irradiation and then transplantation; IR+T+Rg1, irradiation and transplantation following Rg1 administration.

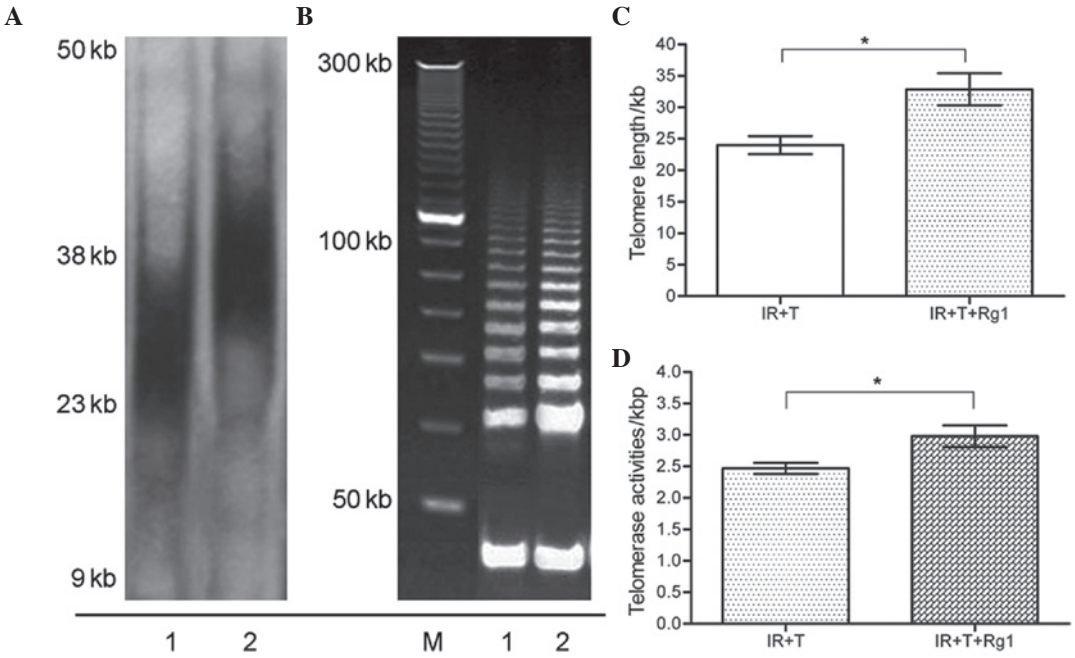

Figure 5. Rg1 functions in telomere elongation and telomerase maintenance of aged Sca-1+ cells in mice. (A) Comparison of telomere length in Sca-1+ cells in the IR+T and IR+T+Rg1 groups of cells from the third transplantation recipient mice. (B) Comparison of the telomerase activities of Sca-1+ cells in the IR+T and IR+T+Rg1 groups of cells from the third transplantation recipient mice. Quantification of respective (C) telomere lengths and (D) telomerase activities of Sca-1+ cells in the different treatment groups. Data are expressed as the mean ± standard deviation (n=3). *P<0.05. Rg1, ginsenoside Rg1; IR+T, irradiation and then transplantation; IR+T+Rg1, irradiation and transplantation following Rg1 administration; M, marker; 1, IR+T; 2, IR+T+Rg1.

an organism and in the regulation of cell senescence, which protects chromosome telomeres from degradation (22). To further confirm that the protective effects of Rg1 on Sca-1+ cells aging is consistent with telomere length compensation, the present study compared the telomerase activities of the Rg1-treated ad control group Sca-1+ cells from third recipient mice. In agreement with the effects of Rg1 on telomere length restoration in the Sca-1+ cells, these results indicated that the telomerase activities of the Sca-1+ cells improved following Rg1 administration, compared with the control group cells in the third recipient mice, as is shown in Fig. 5.

Discussion

In the present study, Rg1 was found to selectively act on aged Sca-1+ cell and normal Sca-1+ cell function to alleviate aging, which was possibly regulated by the *p16-Rb* signaling pathways. These findings were evidenced by an increase in the telomerase activities and telomere lengths of the aged Sca-1+ cells following Rg1 treatment *in vitro*. Secondly, the serial transplantation assay revealed that Rg1 reconstituted the telomerase activities and elongated the telomere lengths of the aged Sca-1+ cells in the recipient mice.

Panax ginseng has traditionally been administered to treat 'deficiency' conditions associated with symptoms, including fatigue, irritability or respiratory tract symptoms in traditional Chinese medicine (23). Rg1, one of the various ginsenosides from *Panax ginseng*, has been demonstrated as a potential anti-aging agent on neural progenitor cells in the dentate gyrus of the hippocampus of normal adult mice and in a model of global ischemia in gerbils (17). Cellular senescence is a stress response, which is characterized by permanent cell proliferation arrest and is triggered by various factors including oncogenes, oxidative stress and persistent DNA damage (24). Loss of immune function and an increased incidence of myeloid leukemia are two of the most clinically significant consequences of aging of hematopoietic cells (25). The anti-aging effect of Rg1 on hematopoietic cell was demonstrated in the present study by the fact that the aging profile of the aged Sca-1$^+$ cells was alleviated following treatment with Rg1. The results also revealed prolonged telomere lengths of the Sca-1$^+$ cells following Rg1 treatment. The results of the present study also demonstrated that high levels of telomerase activities were responsible for stem cell telomere maintenance, which was consistent with a previous report that telomere maintenance is the predominant mechanism underlying the anti-aging phenotype of telomerase-overexpressing transgenic mice (26).

Cells in a state of senescence exhibit specific features, including an enlarged and flattened morphology, increased β-galactosidase activity and cell cycle arrest (27). The activity of SA-β-Gal can be detected at pH 6.0 in several types of cultured cells undergoing replicative and stress-induced senescence (28). It has been used widely as a marker of cellular senescence *in vivo* and *in vitro* (29). This is consistent with the results of the present study, in which the number of positively stained Sca-1$^+$ cells decreased following Rg1 treatment *in vitro*.

Telomere shortening is attributed to the accumulation of DNA single-strand breaks, induced by oxidative damage. Excessive telomere shortening and severe telomere uncapping owing to telomerase deficiency trigger a DNA damage response at the chromosome ends, which subsequently upregulates cell cycle-negative modulators and involves activation of the P16 and Rb proteins (30). The results of the present study demonstrated that the expression levels of the cell cycle negative regulators, P16 and Rb, in the normal and aged Sca-1$^+$ cells were reduced following treatment with Rg1. This implied that the anti-aging effect of Rg1 on the Sca-1$^+$ cells was mediated by cell cycle regulation, which wa involved in telomere and telomerase function. Replicative senescence is another mechanism of senescence that share the same feathers with stress-induced senescence (31). In the present study, the Sca-1$^+$ cells exhibited marked senescence feathers following three serial generation transplantations, indicating that replicative senescence of the Sca-1$^+$ cells was achieved in mice. The aging phenotype of the Sca-1$^+$ cells was retrieved following Rg1 treatment, in a generation-dependent manner.

Taken together, the result of the present study confirmed that Rg1 had protective effects on aged Sca-1$^+$ cells from mice *in vitro* and *in vivo*, in which the *p16-Rb* signaling pathways were responsible for regulating the alleviation of senescence. These findings demonstrate the potential for the anti-aging function of Rg1 on adult stem and progenitor cells, particularly on hematopoietic stem and progenitor cells, to be achieved using external telomerase intervention.

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