Ginsenoside Rg1 protects against Sca-1+ HSC/HPC cell aging by regulating the SIRT1-FOXO3 and SIRT3-SOD2 signaling pathways in a γ-ray irradiation-induced aging mice model

YAN-LONG TANG1, YUE ZHOU1, YA-PING WANG2, YING-HONG HE1, JI-CHAO DING1, YUAN LI1 and CUI-LI WANG1

1Department of Histology and Embryology, Key Laboratory of Cell Biology, Dali University, Dali, Yunnan 671000; 2Laboratory of Stem Cell and Tissue Engineering, Department of Histology and Embryology, Chongqing Medical University, Chongqing 400016, P.R. China

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Abstract. Aging is characterized by a progressive deterioration in metabolic functions. The present study aimed to investigate the antagonistic effects of ginsenoside Rg1 (Rg1) on the γ-ray irradiation-induced aging of mixed hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs). C57BL/6 mice were divided into a control group, a γ-ray irradiation group that served as an aging mouse model, and an Rg1 group. The Rg1 group was treated with Rg1 at dosage of 20 mg/kg/day for 7 days prior to γ-ray irradiation. The aging mouse model was established by exposing the mice to 6.5-Gy γ-ray total-body irradiation. Stem cell antigen 1 positive (Sca-1+) HSC/HPCs isolated from the mice were examined using a senescence-associated β-galactosidase (SA-β-Gal) staining assay. The cell cycle of the HSC/HPCs was examined using flow cytometry. A mixed hematopoietic progenitor cell colony-forming unit (CFU-mix) assay was also conducted. The mRNA and protein expression levels of sirtuin 1 (SIRT1), SIRT3, forkhead box O3 (FOXO3) and superoxide dismutase (SOD2) were measured using western blot and reverse transcription-quantitative PCR assays. The results indicated that Rg1 treatment significantly increased white blood cell, red blood cell and platelet counts in peripheral blood compared with those in the γ-ray irradiation group (P<0.05). However, Rg1 significantly attenuated the senescence of Sca-1+ HSC/HPCs in the γ-ray irradiation aging mice model. The proportion of SA-β-Gal stained HSC/HPCs was significantly decreased and CFU-Mix counts were significantly increased in the Rg1 group compared with the γ-ray irradiation group (P<0.05). Rg1 significantly increased the mRNA and protein levels of SIRT1, SIRT3, FOXO3 and SOD2 in the Sca-1+ HSC/HPCs compared with those in the γ-ray irradiation group (P<0.05). The percentage of Sca-1+ HSC/HPCs arrested at the G1 phase in the Rg1 group was significantly decreased compared with that in the γ-ray irradiation group (P<0.05). In conclusion, the present study indicates that Rg1 exerts anti-aging effects via the regulation of SIRT1-FOXO3 and SIRT3-SOD2 signaling pathways, and triggering the progression of Sca-1+ HSC/HPCs from the G1 phase to the S phase in γ-ray irradiation-induced aging mice.

Introduction

Aging is mainly characterized by a progressive dysfunction of metabolism and various physiological roles, such as maintaining metabolic rate and delaying cell aging (1,2). Clinically, abnormalities of the metabolism and physiological functions can affect the morbidity and mortality of patients. A previous study reported that injury- or damage-associated risk factors are able to cause obvious aging of stem cells (3). Reactive oxygen species (ROS) have been shown to cause injury or damage to cells (4,5).

Recently, radiotherapy has been extensively applied for the treatment of cancers. Although radiotherapy inhibits the proliferation of tumor cells, it also suppresses the growth of hematopoietic stem cells (6). Therefore, the discovery of drugs that could effectively prevent the radiation-induced damage of cells in the hematopoietic system undergoing radiotherapy would be important. Previous studies have investigated the anti-oxidant capacity of drugs by exploring the progression of aging and associated oxidative injuries (7,8); however, studies on the effects of antioxidants on radiation-induced hematopoietic hypofunction are lacking.

Ginsenoside Rg1 (Rg1) is the most active ingredient of Panax ginseng, and is characterized by radiation resistance, anti-aging and anti-oxidation effects (9). A previous study conducted by the present research team showed that Rg1 could attenuate the aging processes of hematopoietic stem cells in an Rg1 group compared with a γ-ray irradiation group (P<0.05). Rg1 significantly increased the mRNA and protein levels of SIRT1, SIRT3, FOXO3 and SOD2 in the Sca-1+ HSC/HPCs compared with those in the γ-ray irradiation group (P<0.05). The percentage of Sca-1+ HSC/HPCs arrested at the G1 phase in the Rg1 group was significantly decreased compared with that in the γ-ray irradiation group (P<0.05). In conclusion, the present study indicates that Rg1 exerts anti-aging effects via the regulation of SIRT1-FOXO3 and SIRT3-SOD2 signaling pathways, and triggering the progression of Sca-1+ HSC/HPCs from the G1 phase to the S phase in γ-ray irradiation-induced aging mice.

Correspondence to: Dr Yue Zhou, Department of Histology and Embryology, Key Laboratory of Cell Biology, Dali University, 2 Hongsheng Road, Dali, Yunnan 671000, P.R. China
E-mail: zhouxille@yeah.net

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cells (HSCs) and hematopoietic progenitor cells (HPCs) (10). Chen et al (11) also reported that Rg1 provided resistance against the radiation-induced aging of mouse HSCs/HPCs. However, the anti-aging mechanism of Rg1 and the associated regulatory molecules have not yet been discovered.

Sirtuins (SIRTs) are highly conserved family of proteins, consisting of seven NAD+-dependent deacetylases, namely SIRT1-7 (12,13). Among these deacetylases, SIRT1 and SIRT3, as the primary deacetylases, participate in cell proliferation, apoptosis, aging and energy metabolism (14,15). A previous study (16) reported that SIRT3 directly regulates ROS production in mitochondria, and further affects cell aging processes. Libert and Guarente (17) reported that SIRT1 serves a critical function in several molecular processes, including inflammation, senescence/aging and intracellular transcription. SIRT1 has also been shown to protect against cellular senescence by deacetylating forkhead box O3 (FOXO3) transcription factors (18). Another previous study demonstrated that SIRT3 could regulate ROS levels by changing the expression of superoxide dismutase 2 (SOD2) (19). A deficiency of SOD2 has also been shown to be associated with aging (20).

In the present study, Rg1 was used to treat a γ-irradiation-induced aging mouse model. In addition, the antagonistic effects of Rg1 on the radiation-induced aging of stem cell antigen 1 positive (Sca-1+) HSC/HPCs were also explored. Furthermore, the regulative role of SIRT1/SIRT3 signaling pathways in the anti-aging effects of Rg1 on Sca-1+ HSCs/HPCs derived from the γ-ray irradiation aging mouse model was also investigated.

Materials and methods

Mice. A total of 90 clean C57BL/6 mice (weighing 20-25 g, 6-8 weeks old, random selection of 43 males and 47 females) were purchased from the Experimental Animal Center of Chongqing Medical University (Chongqing, China). The mice were housed in an environment with a 12-hlight/dark cycle, 40% humidity at room temperature with free access to the food and water. All experiments were approved by the Ethics Committee of the Key Laboratory of Cell Biology (Kunming, China).

Animal grouping. The mice were divided into three groups, namely the control group, γ-ray irradiation group and Rg1 group. The mice in the control group (n=30) were intraperitoneally injected with normal saline and not exposed to γ-ray irradiation. The mice in the γ-ray irradiation group (n=30) were intraperitoneally injected with normal saline for 7 days, followed by exposure to 6.5-Gy γ-ray total-body irradiation. The γ-rays were delivered by a linear accelerator at a dose rate of 57.28 Gy/min (11). The mice in the Rg1 group (n=30) were subjected to the same processes as the γ-ray irradiation group, but with the replacement of normal saline by the same volume of Rg1 at dose of 20 mg/kg/day. The time interval between the final injection and irradiation was 24 h. The specific processes for the treatment of each group were conducted according to a previously published study (11). The Rg1 (cat. no. 060427; purity >95%) was purchased from Jilin Hongjiu Biological Technology Co., Ltd.

Sample preparation. Prior to irradiation treatment, the mice were anesthetized by the intraperitoneal injection of 55 mg/kg pentobarbital. Following the 7 day treatment protocol, mice were sacrificed via the intraperitoneal injection of 150 mg/kg pentobarbital (Altana AG) on day 8. The Sca-1+ HSC/HPCs were harvested using an immunomagnetic separation method as described in a previous study (21). The harvested Sca-1+ HSC/HPCs were used in several experiments, including senescence-associated β-galactosidase (SA-β-Gal) cytochemical staining, flow cytometry (FCM), mixed hematopoietic progenitor cell colony-forming unit (CFU-Mix), reverse transcription-quantitative PCR (RT-qPCR) and western blot assays.

Blood routine analysis. Mice were sacrificed as described above, and blood was collected from the eyeball. Blood routine tests, including white blood cell (WBC), red blood cell (RBC) and blood platelet (PLT) counts were conducted using an XE-2100 hematology analyzer (Sysmex Corporation).

SA-β-Gal staining. Sca-1+ HSC/HPCs were collected on day 7 from every group. The HSC/HPCs were stained using an Senescence β-Galactosidase Staining kit (cat. no. 9860; Cell Signaling Technology, Inc.) according to the manufacturer's instructions. In brief, the purified cells (1x10^6) were washed three times using PBS for 5 min each time, and fixed using 4% paraformaldehyde (Beyotime Institute of Biotechnology) for 10 min at room temperature. Then, the Sca-1+ HSC/HPCs were stained using staining solution at 37°C for 12 h. The cells were centrifuged at 1,000 x g at room temperature for 10 min and cytospin slides were prepared. Subsequently, the slides were sheet-sealed using 70% glycerol (Sigma-Aldrich; Merck KGaA). In order to observe and calculate the percentage of blue-stained positive cells under bright field illumination, ~1x10^4 cells were separated on a slide, and 400 cells on each slide were selected randomly and counted.

CFU-mix culture. The CFU-mix assay was performed according to a previously published study (22) with a few modifications. Briefly, the cells (1x10^4 cells/group) were incubated with DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with horse serum (Gibco; Thermo Fisher Scientific, Inc.), 2-mercaptoethanol (1x10^-4 mol/l; Sigma-Aldrich; Merck KGaA), 3% L-glutamine (Sigma-Aldrich; Merck KGaA), recombinant human erythropoietin (Kyowa Hakko Kirin China Pharmaceutical Co., Ltd.), interleukin-3 (IL-3; Sigma-Aldrich; Merck KGaA) and 2.7% methylcellulose. The components were mixed intensively, seeded into 96-well plates (0.2 ml/well) and then cultured at 37°C with 5% CO2 for 7 days. The CFU-Mix and multiple-differentiation capacities were evaluated according to the CFU-Mix numbers and percentage of Sca-1+ HSC/HPCs.

FCM assay of the cell cycle. Sca-1+ HSC/HPCs were collected from all three groups and washed using PBS. Sca-1+ HSC/HPCs were incubated with 70% iced ethanol overnight, and washed with PBS three times (5 min/time). The Sca-1+ HSC/HPCs were then incubated with bovine pancreatic ribonuclease (cat. no. R2638; Sigma-Aldrich; Merck KGaA;
Table I. Primers used for quantitative PCR.

| Primers | Forward (5'-3') | Reverse (5'-3') | Gene length (bp) | Tm (˚C) |
|---------|----------------|----------------|------------------|---------|
| SIRT1   | AAAATGATGACATCAAGAACG | GCCAATCATGAGATGTTGCTG | 104 | 64 |
| SIRT3   | GCTTCTGCGGTCTTACACAG | CACCCCTGCAGCCATAC | 169 | 66 |
| FOXO3   | CAGTCACCACATGCAGACTATCC | GTCGCTTGAGTGAGTGGTCC | 117 | 68 |
| SOD2    | GAGGCTATCAAGCGCTGATTGTTT | GCAATGGGTCTCTGATTGAGC | 157 | 67 |
| β-actin | GAGACCTTCAACCCCCACACG | ATGTACGCAGATTTCCC | 263 | 61 |

SIRT, sirtuin; FOXO3, forhead box O3; SOD2, superoxide dismutase 2; Tm, melting temperature.

RT-qPCR assay. Total cellular RNAs of Sca-1+ HSC/HPCs were extracted using TRIZol reagent (cat. no. 15596018; Thermo Fisher Scientific, Inc.). ABeyoRTIII First Strand cDND Synthesis kit (cat. no. D7178S; Beyotime Institute of Biotechnology) was used to synthesize cDNAs from the RNA. All processes of the reverse transcription were conducted according to the manufacturer's protocol. The synthesized cDNAs were amplified using SYBR-Green I as the fluoroscopic dye. RT-qPCR was performed using a Fast real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following conditions were used for amplification: 94˚C for 4 min, 94˚C for 20 sec, 60˚C for 30 sec and 72˚C for 30 sec, for 35 cycles. The primers for SIRT1, SIRT3, FOXO3, SOD2 and β-actin are listed in Table I. The relative mRNA expression of the target gene was normalized to that of β-actin using the comparative threshold cycle (ΔΔCq) method (23).

Western blot assay. Proteins were isolated from Sca-1+ HSC/HPCs by lysing cells with lysis buffer (Beyotime Institute of Biotechnology). The concentration of isolated proteins was calculated with a BCA protein quantification kit (Beyotime Institute of Biotechnology). Equal amounts of protein (0.2 µg) were separated using 15% SDS-PAGE (Sigma-Aldrich; Thermo Fisher Scientific, Inc.). The western blot bands were visualized using horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2,000; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. The PVDF membranes were then washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2,000; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Finally, the stained PVDF membranes were washed and incubated with goat anti-rabbit IgG (1:2,000; Santa Cruz Biotechnology, Inc.) at room temperature in the dark. Finally, the stained PVDF membranes (Pierce; Thermo Fisher Scientific, Inc.) were separated using 15% SDS-PAGE (Sigma-Aldrich; Thermo Fisher Scientific, Inc.) and electrotransferred onto PVDF membranes (Pierce; Thermo Fisher Scientific, Inc.). The western blotting density was qualified using the NIH ImageJ software version 1.46 (National Institutes of Health).

Statistical analysis. Data are presented as the mean ± standard deviation. Data were analyzed using SPSS software 11.0 (SPSS, Inc.). All data were obtained from at least three independent tests or experiments. Tukey's post hoc test was used following ANOVA to compare continuous data among multiple groups. P<0.05 was considered to indicate statistical significance.

Results

Rg1 improves peripheral blood levels in γ-ray irradiated aging mice. In order to investigate Sca-1+ HSC/HPC-induced hematopoietic functions, the WBC, RBC and PLT levels in the peripheral blood of the mice were examined. The results indicated that the WBC, RBC and PLT counts in the γ-ray irradiation group were significantly decreased compared with those in the control group (P<0.01; Table II). Notably, Rg1 treatment significantly increased the WBC, RBC and PLT counts compared with those in the γ-ray irradiation group (P<0.05; Table II). However, the WBC, RBC and PLT counts in the Rg1 group were significantly lower compared with those in the control group (P<0.01; Table II). Notably, Rg1 treatment significantly increased the WBC, RBC and PLT counts compared with those in the control group (P<0.01; Table II).

Rg1 affects the senescence of Sca-1+ HSC/HPCs in γ-ray irradiated aging mice. SA-β-Gal is an extensively applied
biodmarkerm for aging cells (24). A decreased CFU-Mix formation capacity is also an evauative marker for the senescence of HSCs (25). Therefore, an SA-β-Gal staining assay and CFU-Mix formation assay were performed to evaluate the effects of Rg1 on the senescence of Sca-1^+ HSC/HPCs.

The results showed that γ-ray irradiation induced a significant increase in the percentage of SA-β-Gal stained Sca-1^+ HSC/HPCs (P<0.01; Fig. 1A) and a significant reduction in the CFU-Mix counts of Sca-1^+ HSC/HPCs, compared with the control group (P<0.01; Fig. 1B). In the Rg1 group, the proportion of SA-β-Gal stained HSC/HPCs was significantly decreased (Fig. 1A; P<0.05) and the CFU-Mix count was significantly increased (Fig. 1B; P<0.05), compared with the respective values in the γ-ray irradiation group. However, the percentage of SA-β-Gal stained HSC/HPCs and CFU-Mix numbers in the γ-ray irradiation group were also significantly higher and significantly lower, respectively, compared with those in the control group (Fig. 1; P<0.05).

Rg1 increased the mRNA levels of SIRT1/SIRT3 signaling molecules in Sca-1^+ HSC/HPCs. Cell proliferation-associated SIRT1 and SIRT3 molecules were examined using RT-qPCR. Compared with the control group, SIRT1 (Fig. 2A) and SIRT3 (Fig. 2B) mRNA levels in the γ-ray irradiation group were significantly decreased (both P<0.01). However, Rg1 treatment (Rg1 group) significantly upregulated SIRT1 (Fig. 2A) and SIRT3 (Fig. 2B) mRNA levels compared with those in the γ-ray irradiation group (P<0.05).

The downstream molecule of SIRT1 (FOXO3) and the downstream molecule of SIRT3 (SOD2) were also examined. The results indicated that FOXO3 (Fig. 2C) and SOD2 (Fig. 2D) mRNA levels were significantly decreased in the γ-ray irradiation group compared with the control group (both P<0.01). However, Rg1 treatment significantly increased the mRNA levels of FOXO3 (Fig. 2C) and SOD2 (Fig. 2D) compared with those in the γ-ray irradiation group (both P<0.05).

Rg1 impacted the cell cycle distribution of Sca-1^+ HSC/HPCs in the γ-ray irradiation aging mouse model. Previous studies (26,27) reported that SIRT1 and SIRT3 participate in cell proliferation and cell cycle regulation. Furthermore, HSCs undergoing aging are arrested at the G1 stage (28). Therefore, the cell cycle distribution of Sca-1^+ HSC/HPCs was analyzed using FCM. The significant G1 phase arrest of Sca-1^+ HSC/HPCs was observed, and the percentages of cells in the S and M phases were significantly decreased in the γ-ray irradiation and Rg1 groups, compared with those in the control group (Fig. 4; all P<0.05). However, when compared with the γ-ray irradiation group, the SIRT1, SIRT3, FOXO3 and SOD2 expression levels in the Rg1 group were significantly increased (Fig. 3B; all P<0.05).
The proliferative index (PI; S + G2/M) in the γ-ray irradiation group was significantly decreased compared with that in the control group (Fig. 4; P<0.01). However, the PI of Sca-1+ HSC/HPCs in the Rg1 group was significantly increased compared with that in the γ-ray irradiation group (Fig. 4; P<0.05).

Discussion

Scientific studies have demonstrated that the Chinese herbal drug ginseng is characterized by anti-injury, anti-aging and anti-oxidant functions, and the ability to enhance immunity (29,30). Zhu et al. (31) found that Rg1 could prolong the life of mice and delay the aging processes of human lung fibroblasts. In the aging processes of cells, HSC/HPCs in the hematopoietic system exhibit progressive and morphological changes (32). Zhou et al. (33) reported that Rg1 delays the tert-butyl hydroperoxide-induced aging of HSCs.

A previous study (34) reported that radiation induces damage or injury in HSC/HPCs, causes cells to undergo apoptosis and senescence, and reduces the hematological reconstitution function of HSC/HPCs. Chen et al. (11) found that Rg1 promotes the proliferation of hematopoietic cells; however, further evaluation of the mechanism is required. The present study showed that γ-ray irradiation significantly decreased the WBC, RBC and PLT counts of mice compared with those in un-irradiated controls; however, Rg1 was able to attenuate these reductions in blood cell levels. Therefore, the present findings confirm that Rg1 prevents irradiation-induced hematopoietic dysfunction.

It is speculated that the improvement of hematopoietic function observed in the mice might be triggered by increased levels of Sca-1+ HSC/HPCs. SA-β-Gal accumulates in aging cells and reflects the dysfunction of cells (24). Furthermore,
the ability of mixed hematopoietic progenitor cells to form colonies decreases following the regression of self-renewal potential in aging Sca-1⁺ HSC/HPCs (35). Therefore, the aging or senescence of Sca-1⁺ HSC/HPCs was evaluated using the SA-β-Gal method and CFU-Mix assay in the present study. The results demonstrated that the percentage of SA-β-Gal stained HSC/HPCs was significantly increased and CFU-Mix counts were significantly decreased following γ-ray irradiation compared with those in the control group. Notably, Rg1 treatment significantly attenuated the γ-ray irradiation-induced and aging/senescence-associated changes. These findings are consistent with a previous study (11), which reported that Rg1 delays the irradiation-induced senescence of Sca-1⁺ HSC/HPCs. Therefore, the present data suggest that Rg1 is able to inhibit the aging of Sca-1⁺ HSC/HPCs.

At present, several signaling pathways have been demonstrated to participate in cell senescence or aging; these include SIRT1 and SIRT3 pathways, which may inhibit cellular senescence by regulating the cell cycle (26,27,36). SIRT3 upregulates the self-renewal and multi-differentiation capacity of HSCs, and further delays their aging processes (37). SIRT3 also enhances the activity of SOD2 by triggering deacetylation, which plays an important role in the negative regulation of organism aging (38). Therefore, SIRT3 and SOD2 expression were examined in the present study. The results indicated that irradiation significantly induced the downregulation of SIRT3 and SOD2 in Sca-1⁺ HSC/HPCs. This suggests that irradiation might initiate the progression of aging and oxidative stress. SOD2 is also considered to be an important anti-oxidant defense enzyme; therefore, it is speculated that γ-ray irradiation might result in the oxidative damage of Sca-1⁺ HSC/HPCs via the reduction of SOD2 activity. However, the results of the present study revealed that Rg1 significantly inhibited the irradiation-induced downregulation of SIRT3 and SOD2 expression. This suggests that Rg1 is able to inhibit the aging of Sca-1⁺ HSC/HPCs and increase SOD2 activity, which is critical to prevent Sca-1⁺ HSC/HPCs from undergoing irradiation-induced aging or oxidative stress.

It has previously been reported that SIRT1 can delay cardiovascular and neuron aging (39). Morris et al (40) reported that FOXO3 is an important gene for human longevity. It is well known that the SIRT1 gene can interact with FOXO3 and regulate FOXO3 expression in the mitochondria of cells (41). Therefore, it is hypothesized that the effects of SIRT1 on aging might be triggered by FOXO3 expression. Therefore, SIRT1 and FOXO3 expression were examined in the present study, and it was found that irradiation treatment significantly downregulated SIRT1 and FOXO3 levels in Sca-1⁺ HSC/HPCs. However, Rg1 treatment significantly attenuated the irradiation-induced SIRT1 and FOXO3 downregulation. These results suggest that Rg1 may also suppress the aging of Sca-1⁺ HSC/HPCs via the activation of SIRT3 and FOXO3 expression.

A previous study (26) reported that SIRT3 overexpression disrupts mitochondrial proteostasis, induces cell cycle arrest and inhibits the proliferation of cells. In another study, SIRT1 was reported to contribute to delay of the epithelial cell cycle in diabetic corneas (42). Therefore, the cell cycle status of Sca-1⁺ HSC/HPCs was investigated in the present study to determine
the proportion of cells in the G1, S, G2 and M phases. The results indicated that irradiation triggered cell cycle arrest at the G1 phase and that cell senescence was induced. However, as discussed above, Rg1 treatment significantly increased SIRT1 and SIRT3 expression in irradiated Sca-1+ HSC/HPCs. The increased SIRT1 and SIRT3 expression may have inhibited cells from arresting in the G1 phase and suppressed cell senescence. Irradiation-induced arrest of the cell cycle at the G1 phase, without entry to the S phase, is mainly induced by the gradual loss of mitosis reactivity and DNA synthesis activity in aging cells (28). The cell cycle results in the present study suggest that Rg1 treatment significantly attenuated the irradiation-induced G1 arrest by regulating the expression of SIRT1 and SIRT3. This may have triggered the progression of cells from the G1 phase to the S phase, and thereby delayed the senescence of Sca-1+ HSC/HPCs.

In conclusion, Rg1 decreased the percentage of SA-β-Gal stained Sca-1+ HSC/HPCs, and increased the ability of HSC/HPCs to form colonies. These results suggest that Rg1 treatment attenuates γ-ray irradiation-induced aging in a mouse model. Rg1 may exert anti-aging effects via the enhancement of SOD2 activity and reduction of SIRT3, SIRT1 and FOXO3 expression. This study provides a promising hypothesis for the mechanism by which Rg1 application delays the aging of Sca-1+ HSC/HPCs.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

YLT and YZ designed this study and wrote the manuscript. YLT, YZ, YPW, YHH and JCD performed the experiments or tests. YL and CLW analyzed the data. CLW reviewed the literature. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were approved by the Ethics Committee of the Key Laboratory of Cell Biology (Kunming, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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