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Original Article

The aging-delay effects of ginsenoside Rg1 on hemopoietic stem and progenitor cell are related with changes of cell cycle regulating molecule expression

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Abstract: Objective: to study the aging-delay effect and the potential mechanism of ginsenoside Rg1 in the process of hemopoietic stem cell/hemopoietic progenitor cell sequential transplantation. Method: isolate Sca-1$^+$HSC/HPC from male donor mice. Female recipient mice were divided into four groups after ^{60}Co γ-radiation with fatal dose, control group, aging model group, Rg1 anti-aging group, and Rg1 aging-delay group. We performed the sequential transplantation of Sca-1$^+$HSC/HPC for three generations to construct the in vivo aging mode. Real-time fluorescence quota PCR was used in the detection of the expression of SRY gene in the marrow of the recipients, to ascertain the source of hemocyte, the survival time of the recipients and the hemogram of peripheral blood was observed to confirm the hematopoietic reconstitution and the improvement which was caused by Rg1; CFU-mix, cell cycle analysis and senescence-associated β-galactosidase staining were employed to analysis the biological characters of senescent Sca-1$^+$HSC/HPC cells and the in vivo regulation of Rg1 in this process. Real-time fluorescence quota PCR was used to detect the expression levels of Sca-1$^+$HSC/HPC p16^{INK4a}, p19^{Arf}, p53, p21$^{Cip1/Waf1}$ mRNA, and western blotting was used to detect the protein levels of Sca-1$^+$HSC/HPC P16^{INK4a}, CDK4, CyclinD, P21$^{Cip1/Waf1}$, CDK2, CyclinE in recipients. Results: the hemocyte of recipients was from male donors, the 30 d survival rate was dramatically declined after cell sequential transplantation. The hemogram of peripheral blood recovered slowly, and Sca-1$^+$HSC/HPC of these recipients were arrested in G_1 phase, which resulted in the CFU-mix decrease and the positive rate of SA-β-Gal. Every generation in the Rg1 anti-aging group, and Rg1 aging-delay group showed higher 30d survival rate, CFU-mix, WBC, HCT, PLT, and lower rates of cells in G_1 phase and SA-β-Gal positive cells than their control in the aging model group. When treated with Rg1, the mRNA level of Sca-1$^+$HSC p16^{INK4a}, p19^{Arf}, p53, p21$^{Cip1/Waf1}$ and the protein level of P16^{INK4a}, P21$^{Cip1/Waf1}$, CyclinD1 were down-regulated, the protein level of CDK4, CDK2, CyclinE were up-regulated. These effects were obvious in the Rg1 aging-delay group comparing with the Rg1 anti-aging group. Conclusion: Ginsenoside Rg1 plays an anti-aging role in the process of Sca-1$^+$HSC/HPC sequential transplantation, it has a better effect of aging-delay than anti-aging. The underlying mechanism may relate with its regulation of cell cycle regulating molecule expression.

Keywords: Ginsenoside Rg1, hemopoietic stem cell/hemopoietic progenitor cell, aging, cell cycle regulating molecule

Introduction

Ginseng has functions of anti-aging, antioxidant, promoting cell proliferation and differentiation. In recent studies, ginsenoside Rg1 was reported to has an anti-aging effect, however, the mechanisms are not clarified [1, 2]. In this research, we performed sequential transplantation of Sca-1$^+$HSC/HPC to construct an in vivo aging model. Then we treated this model with Rg1 to investigate whether Rg1 has anti-aging effect on Sca-1$^+$HSC/HPC and to investigate the regulation mechanism. Our results showed that Rg1 can enhance the self renewing and multiple differentiative potent of Sca-1$^+$HSC/HPC. In vivo experiments showed an anti-aging ability of Rg1. Rg1 played the anti-aging role through the downregulating the expression of p16^{INK4a}, p19^{Arf}, p53, p21$^{Cip1/Waf1}$ mRNA and the protein level of P16^{INK4a}, P21$^{Cip1/Waf1}$, and CyclinD1, and, on the other hand, upregulating the protein level of CDK4, CDK2, and CyclinE.

The regulation of cell cycle regulating molecule expression may be one of the anti-aging mechanisms of Rg1 treatment on Sca-1$^+$HSC/HP.

Material and methods

Material and reagents

Laboratory animals C57BL/6 mice (clean class) 6-8 weeks old, male and female, 20-25 g, offered by Chongqing laboratory animal center. Animal Epidemic Prevention Certificate number: SCXK (Yu) 2007-0001.

Reagent

Ginsenoside Rg1 (Jilin Hongjiu bio technology Co, purity >95%), IMDM, FBS, horse serum (Gibco), Anti- Sca-1$^+$ Micro Bead Kit (Miltenyi), SA-β-gal Staining Kit (Cell Signaling), L-glutamine, methylcellulose (Sigma), rhGM-CSF, rhEPO (Qilinkunpeng biological pharmaceutical Co.LTD), PCR primers (Invitrogen), sybr green I (Biorad), fluorescence quota PCR kit (TaKaRa), RIPA buffer (Cell Signaling), primary antibodies of $P16^{INK4a}$, $P21^{Cip1/Waf1}$, CDK4, CDK2, CyclinD1, and CyclinE, and goat anti-rabbit IgG were all from Santa Cruz.

Methods

Isolation, purification, and identification of male Sca-1$^+$HSC/HPC Sca-1$^+$ cells were isolated and purified according to the instruction of magnetic beads sorting kit and the references [3, 4], and then sorted by FACS.

Female recipient hematopoietic failure model construction and group dividing female recipients were placed in an organic glass illuminating incubator, exposed in ^{60}Co γ-radiation with fatal dose (8.5 Gy), distance: 75 cm, dose: 57.28c Gy/min. group dividing: 1) control group, 4-6 h after radiation, the recipients were injected with 0.2 ml PBS into caudal vein; 2) aging model group, 4-6 h after radiation, the recipients were transplanted with 0.2 ml Sca-1$^+$HSC/HPC cell suspension into caudal vein, which contained 2×10^4 Sca-1$^+$HSPC; 3) Rg1 anti-aging group, 4-6 h after radiation, each recipient was transplanted with the same dose of Sca-1$^+$HSC/HPC, and then treated with 20 mg/kg/day Rg1 for 30 days; 4) Rg1 aging-delay group, each mouse was pretreated with 20 mg/kg/day Rg1 for 10 days, after radiation, mice were treated as the Rg1 anti-aging group. 30 days after transplantation, donor's Sca-1$^+$HSC/HPC cells were isolated from bone marrow cells of the first generation recipients. These Sca-1$^+$HSC/HPC cells were injected into the second generation female recipients, after three sequential transplantation, each generation of Sca-1$^+$HSC/HPC of all the four groups were isolated for biological detections.

Sry gene detection of bone marrow cells after the hematopoietic reconstitution of recipients' total RNA of recipients' bone marrow were extracted and 260/280 was analyzed by ultraviolet spectrophotometer. The integrity of RNA was analyzed by 1% argose gel. Primers were designed as follows: Sry F: GAAAAGCCTTACAGAAGCCGA, R: GTATGTGATGGCATGTGGGTTC. Total RNA was reverse transcripted into cDNA. The fluorescence quota PCR system was 2× PCR buffer 25 μl, 25 pmol/μl Sry-F 0.6 μl, 25 pmol/μl Sry-R 0.6 μl, cDNA 1 μl, Sybr green I 0.3 μl, DEPC-treated H_2O 22.5 μl. the reaction condition was 94°C 4 min; 94°C 20 s, 60°C 30 s, 72°C 30 s, the reaction insisted of 35 cycles. The Fluorescent signal was detected at 72°C. Each sample contained 3 parallel tubes.

Detection of the survival time and the hemogram of peripheral blood of the recipients

The recipients' survival time and survival rate of 30 days after transplantation were observed.

On the 30th day after transplantation, blood sample was taken from eyes of each recipient. Blood samples were treated with sodium citrate. blood routine of the samples (WBC, HCT, PLT) were analyzed by blood cell analyzer in the clinical lab of the first affiliated hospital of Chongqing medical University.

Relative index detection of recipient mice Sca-1$^+$HSC/HPC in vitro aging

Senescence-associated β-galactosidase staining recipients' Sca-1$^+$HSC/HPC in every generation of each group were isolated, washed with PBS for 2 times, cells were treated as the instruction of SA-β-Gal Staining Kit, cells were incubated at 37°C, CO_2-free condition for 12 h for staining. After the incubation, cells were cytofuged. Cell density was 1×10^4 per slide. Slides were fixed with 70% glycerol and observed under microscope. 400 cells were ran-

Table 1. The expression of sry gene in the recipients' bone marrow ($\bar{x}$±s, n=3)

	1st generation			2nd generation			3rd generation		
	Normal male	Female recipient	Normal female	Normal male	Female recipient	Normal female	Normal male	Female recipient	Normal female
$2^{-\Delta Ct}$	1.74±0.03[1)]	1.06±0.03[1)]	0.008±0.0006	1.67±0.03[1)]	0.94±0.01[1)]	0.005±0.0007	0.65±0.02[1)]	0.58±0.02[1)]	0.003±0.0007

[1)]*P*<0.01 when compared with normal female mice.

dom counted for each slide, the percentage of positive cells was figured out.

CFU-Mix culture the culture was performed according to the references [5, 6] and some improvements were made. 1×10^{-4} mol/L beta mercaptoethanol, 3% L-glutamine, horse serum, rhEPO, IL-3, rhGM-CSF were mixed with 1×10^4 ca-1$^+$HSC/HPC of each sample, then 2.7% methylcellulose were mixed in sequence, and the total volume was adjusted to 2 ml. Then cells were planted in 96-well dishes and incubated in the incubator for 7 days (37°C, 5% CO_2). The cell colony-forming and multiple differentiative abilities were estimated according to the numbers of Sca-1$^+$HSC/HPC planted at the beginning and the CFU-Mix colonies formed.

Flow cytometry for the cell cycle analysis Sca-1$^+$HSC/HPC of each sample was isolated and fixed with 70% ice-cold ethanol over night. After that, cells were incubated with 100 μl RNase, at 37°C for 30 min. samples were stained by PI for 30 min, then the number of cells distributing in each phase of the cell cycle were analyzed by flow cytometry.

The mRNA level of p16^{INK4a}, p19^{Arf}, p53, p21$^{Cip1/Waf1}$ were detected by fluorescence quota PCR

Sca-1$^+$HSC/HPC of every generation were isolated on the 30th day after transplantation, total mRNA was extracted and reverse transcripted into cDNA the reaction system was as follows, 2× RT buffer 10 μl, random primers 100 pmol/μl, RT-mix 1 μl, template RNA 5 μl, DEPC treated H_2O 3 μl. Reaction condition: 25°C 10 min, 40°C 60 min, 85°C 5 min. The real-time fluorescence quota PCR system was 2× PCR buffer 25 μl, 25 pmol/μl p16INK4a/p19Arf/p53/p21-Cip1/Waf1/β-action forward primer/reverse primer 0.6 μl, cDNA 1 μl, Sybr green I 0.3 μl, DEPC treated H_2O 22.5 μl. Reaction condition: 94°C 4 min; 94°C 20 s, 60°C 30 s, 72°C 30 s, 35 cycles; The fluorescent signal was detected at 72°C. every sample contained 3 parallel tubes.

The protein levels of Sca-1$^+$HSC/HPC P16^{INK4a}, CDK4, CyclinD1, P21$^{Cip1/Waf1}$, CDK2, CyclinE were detected by western blotting. Sca-1$^+$HSC/HPC of every generation were isolated on the 30th day after transplantation, total protein was extracted and the protein content was determined by BCA kit. Samples with the same total protein concentration (50 μg/ lane) were loaded on the SDS-PAGE. When transited to the PVDF membrane, the membrane was blocked with 5% fat-free milk for 2 h, and incubated with primary antibodies of P16^{INK4a}, CDK4, and cyclinD1 (1:500 or 1:800 for the internal control) at 4°C overnight. After thorough wash of TBST, the membranes were incubated with goat anti rabbit IgG HRP-conjunct secondary antibody (1:5000), at room temperature for 2 h, washed with TBST for 3 times the membranes were developed by ECL and then exposed in the gel imaging system.

Statistical analyses

Data was showed as average ± standard deviation ($\bar{x}$±s); data was analyzed by SAS 10.1 for factorial design and single factor analysis variance, multiple comparisons were performed with LSD. It was considered that there were statistical differences when *P*<0.05.

Results

The detection of sry gene after hematopoietic reconstitution

There is no sry gene expression in normal female mouse bone marrow. After transplantation, sry gene expression can be detected in all of the 1st, 2nd, and 3rd generations of recipients' bone marrow, and however, there were statistical differences comparing with normal male mice (**Table 1**).

The peripheral blood changes of recipients and the 30 days survival rate observation

As the transplantation generation increased, the hematopoietic reconstitution ability of

Table 2. The phase changes of Sca-1$^+$HSC/HPC in peripheral blood of each group ($\bar{x}\pm s$, n=6)

	1st generation			2nd generation			3rd generation		
	Aging group	Rg1 anti-aging group	Rg_1aging-delay group	Aging group	Rg1 anti-aging group	Rg_1aging-delay group	aging group	Rg1 anti-aging group	Rg_1aging-delay group
WBC (10^9/L)	5.43±0.48	6.14±0.37[1)]	6.74±0.26[1),3)]	3.71±0.18[1)]	4.58±0.16[2),3)]	5.10±0.13[2),4)]	2.9±0.09[1),2)]	3.50±0.20[4),5)]	3.90±0.08[5),6)]
HCT (%)	36.77±9.76	43.1±10.68[1)]	47.18±12.52[1),3)]	30.69±9.28[1)]	35.53±10.10[2),3)]	38.55±11.29[2),4)]	22.42±5.40[1),2)]	25.48±6.94[4),5)]	28.19±5.55[5),6)]
PLT (10^9/L)	491.33±79.16	520.92±131.55[1)]	573.83±74.48[1),3)]	374.46±65.64[1)]	421.13±104.87[2),3)]	447.35±85.50[2),4)]	308.37±51.23[1),2)]	332.12±53.20[4),5)]	353.21±85.76[5),6)]

[1)]when comparing with the 1st generation of aging group, P<0.01; [2)]when comparing with the 2nd generation of aging group P<0.05; [3)]when comparing with the 1st generation of anti-aging group P<0.05; [4)]when comparing with the 2nd generation of anti-aging group P<0.05; [5)] when comparing with the 3rd generation of aging group P<0.05; [6)]when comparing with the 3rd generation of anti-aging group P<0.05.

Table 3. The 30-day survival rate of recipients in each group

	1st generation				2nd generation			3rd generation		
	Control	Aging group	Rg1 antiaging group	Rg1 aging-delay group	Aging group	Rg1 antiaging group	Rg1 aging-delay group	Aging group	Rg1 antiaging group	Rg1 aging-delay group
Survival rate	0	73.3±16.3	83.3±15.1[1)]	80.0±12.6[1)]	60.0±17.9[1)]	70.0±10.9[2)]	66.7±10.3[2)]	50.0±16.7[1),2)]	56.7±15.1[3)]	60.0±12.6[3)]

[1),2)]as same as **Table 2**; [3)]when comparing with the 3rd generation of aging group P<0.05.

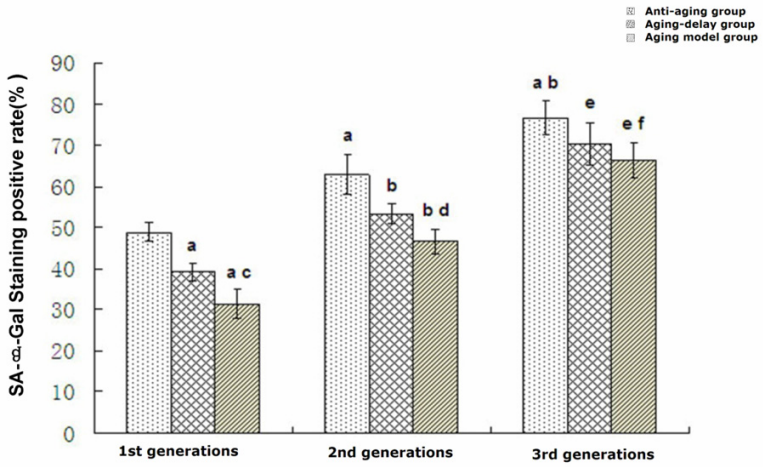

Figure 1. The effect of Rg1 on the Sca-1⁺HSC/HPC SA-β-Gal Staining positive rate. a. When comparing with the 1st generation of aging group, $P<0.01$; b. When comparing with the 2nd generation of aging group $P<0.05$; c. When comparing with the 1st generation of anti-aging group $P<0.05$; d. When comparing with the 2nd generation of anti-aging group $P<0.05$; e. When comparing with the 3rd generation of aging group $P<0.05$; f. When comparing with the 3rd generation of anti-aging group $P<0.05$.

recipients decreased, the recovery of peripheral blood WBC, HCT, and PLT delayed in each phase. In every generation, WBC, HCT, and PLT of Rg1 anti-aging and aging-delay group were higher than aging group of the same generation; what's more, the Rg1 aging-delay group was higher than the anti-aging group (**Table 2**). In control group, all these recipients died 7~15 days after transplantation. In every Sca-1⁺HSC/HPC transplantated group, recipients lived longer than 20 days. As the transplantation generation increased, the survival time shortened. The survival time of Rg1 anti-aging and aging-delay group were longer than aging group of the same generation; however, there were statistical differences between the two groups (**Table 3**).

The effect of Rg1 on the Sca-1⁺HSC/HPC SA-β-Gal Staining positive rate

Blue granules can be seen in the cytoplasma of SA-β-Gal positive cells, while negative cell cannot be stained. As the transplantation generation increased, the positive rate of Sca-1⁺HSC/HPC SA-β-Gal staining in every generation of recipients increased, the positive rate of Rg1 anti-aging and aging-delay group were lower than aging group of the same generation; what's more, the Rg1 aging-delay group was lower than the anti-aging group (**Figure 1**).

The effect of Rg1 on the Sca-1⁺HSC/HPC CFU-Mix forming ability

The numbers of colonies which were formed by the 2nd and 3rd generations of aging group after the transplantation were 9.07±2.06, and 2.31±1.38/10^4 Sca-1⁺HSC/HPC which were significantly lower than the 1st generation of aging group (10.23±3.11/10^4 Sca-1⁺HSC/HPC ($P<0.05$)). The numbers of colonies which were formed by the 1st generations of anti-aging group and aging-delay group were 15.23±1.36, 17.15±4.14/10^4 Sca-1⁺HSC/HPC which were higher than the 1st generation of aging group ($P<0.05$). The numbers of colonies which were formed by the 2nd generations of anti-aging group and aging-delay group were 10.15±4.06, 13.08±2.93/10^4 Sca-1⁺HSC/HPC which were higher than the 2nd generation of aging group ($P<0.05$). The numbers of colonies which were formed by the 3rd generations of anti-aging group and aging-delay group were 4.85±2.03, 6.15±2.27/10^4 Sca-1⁺HSC/HPC which were higher than the 3rd generation of aging group ($P<0.05$).

The effect of Rg1 on Sca-1⁺HSC/HPC cell cycle of the recipients

As the transplantation generation increased, Sca-1⁺HSC/HPC arrested in G_0/G_1 phase were significantly increased; proliferation index (PI) ($S+G_2/M$) was significantly lower. Compared with aging group, Rg1 anti-aging group and aging-delay group had a lower G_0/G_1 cell number and a higher PI. What' more, these indexes of aging-delay group were better than the anti-aging group (**Table 4**).

The effect of Rg1 on the expression of Sca-1⁺HSC/HPC $p16^{INK4a}$, $p19^{Arf}$, p53, $p21^{Cip1/Waf1}$ mRNA

As the transplantation generation increased, the expression of Sca-1⁺HSC/HPC $p16^{INK4a}$, $p19^{Arf}$, p53, $p21^{Cip1/Waf1}$ mRNA was enhanced. In

Table 4. The effect of Rg1 on Sca-1$^+$HSC/HPC cell cycle of the recipients (n=6, $\bar{x}\pm s$)

	1st generation			2nd generation			3rd generation		
	Aging group	Rg_1 anti-aging group	Rg_1 aging-delay group	Aging group	Rg_1 anti-aging group	Rg_1 aging-delay group	Aging group	Rg_1 anti-aging group	Rg_1 aging-delay group
G_0/G_1	64.19±7.83	56.58±3.99[1)]	52.41±4.97[1),3)]	76.74±5.88[1)]	69.49±5.57[2)]	61.41±7.55[2),4)]	86.05±7.22[1),2)]	77.77±6.57[5)]	72.03±7.23[5),6)]
G_2/M	11.78±1.75	12.23±1.16[1)]	12.46±2.22[1),3)]	7.55±2.08[1)]	9.57±2.37[2)]	10.49±1.54[2),4)]	4.79±2.57[1),2)]	7.35±2.75[5)]	7.80v2.52[5),6)]
S	24.03±7.32	31.31±3.84[1)]	35.13±3.31[1),3)]	15.71±4.14[1)]	20.94±3.82[2)]	28.11±6.21[2),4)]	9.17±5.03[1),2)]	15.21±3.53[5)]	20.17±5.36[5),6)]
PI	35.81±7.83	43.54±3.96[1)]	47.59±4.97[1),3)]	23.27±5.88[1)]	30.51±5.57[2)]	38.59±7.55[2),4)]	13.96±7.22[1),2)]	22.23±6.57[5)]	27.97±7.23[5),6)]

[1)]when comparing with the 1st generation of aging group, $P<0.01$; [2)]when comparing with the 2nd generation of aging group $P<0.05$; [3)]when comparing with the 1st generation of anti-aging group $P<0.05$; [4)]when comparing with the 2nd generation of anti-aging group $P<0.05$; [5)]when comparing with the 3rd generation of aging group $P<0.05$; [6)] when comparing with the 3rd generation of anti-aging group $P<0.05$.

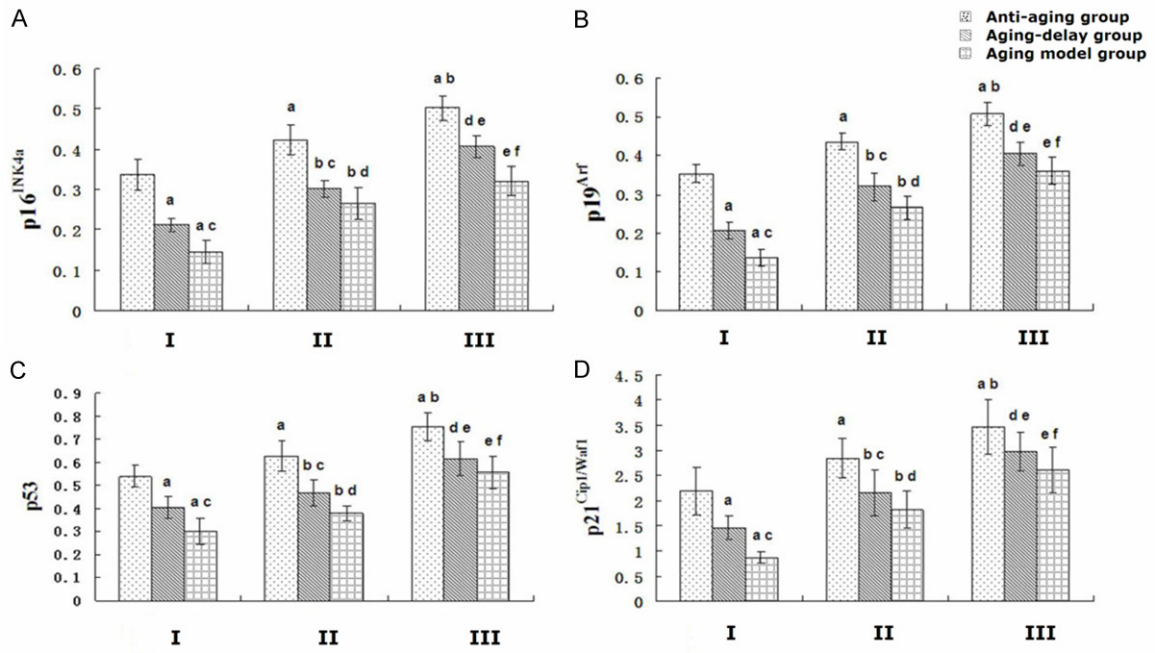

Figure 2. Effect of Rg1 on expretion of $p16^{INK4a}$, $p19^{Arf}$, p53, $p21^{Cip1/Waf1}$ mRNA to Sca-1$^+$HSC/HPC of receptor mice. A. When comparing with the 1st generation of aging group, $P<0.01$; B. When comparing with the 2nd generation of aging group $P<0.05$; C. When comparing with the 1st generation of anti-aging group $P<0.05$; D. When comparing with the 2nd generation of anti-aging group $P<0.05$; E. When comparing with the 3rd generation of aging group $P<0.05$; F. when comparing with the 3rd generation of anti-aging group $P<0.05$.

both of the Rg1 anti-aging group and the Rg1 aging-delay group, the expressions were lower than the aging group, and the aging-delay group were lower than the anti-aging group (**Figure 2**).

The effect of Rg1 on the expression of Sca-1$^+$HSC/HPC cell cycle regulation protein $P16^{INK4a}$, CyclinD1, CDK4, $P21^{Cip1/Waf1}$, CyclinE, CDK2

As the transplantation generation increased, the expression level of Sca-1$^+$HSC/HPC $P16^{INK4a}$, CyclinD1 was up-regulated, while CDK4 was down-regulated. In every generation, the Rg1 anti-aging group and the aging-delay group showed a lower expression level of Sca-1$^+$HSC/HPC $P16^{INK4a}$, and CyclinD1 than the aging group; while the aging-delay group was lower than the anti-aging group. On the other hand, the Rg1 treated groups showed higher level of CDK4 level than the aging group, and aging-delay group was even higher than the anti-aging group (**Figures 3** and **4**).

Discussion

Aging is the irreversible, comprehensive, and gradual degeneration of cells, tissues, or organs in the structure and function. Previous studies showed that the numbers and potent of

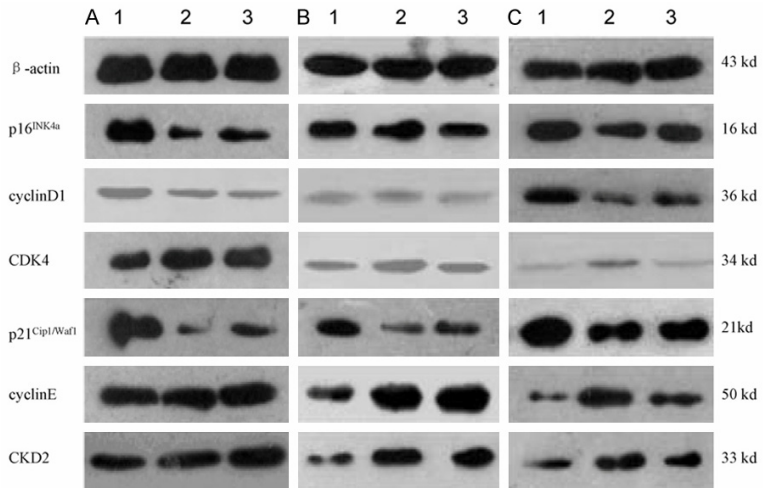

Figure 3. The effect of Rg1 on the expression of Sca-1$^+$HSC/HPC cell cycle regulation protein P16^{INK4a}, CyclinD1, CDK4, P21$^{Cip1/Waf1}$, CyclinE, CDK2. (Western blot). A. 1st generation; B. 2nd generation, C. 3rd generation. 1, aging group, 2, anti-aging group, 3, aging-delay group.

HSC/HPC underwent a gradual degeneration, though the elementary composition of hemopoietic system was maintained in the procession, that is to say, hemopoietic stem cell grow old [7, 8]. There were relationships between the senescence of HSC/HPC and the degeneration of body, the senescence of HSC/HPC result in loss of normal regulation of erythrocyte, immunity abnormality, and increased rates of tumor. What's more, the procession was closely related with senile leukemia and aplastic anemia [9, 10]. So the study on the biologic mechanisms of the senescence of HSC/HPC and effective ways to delay the senescence of HSC/HPC may have important significances on prolonging life and senile ill prevention and cure.

We used the Sca-1$^+$HSC/HPC sequential transplantation experiment to construct a Sca-1$^+$HSC/HPC aging model. We found that in the sequential transplantation for three generations, the Sca-1$^+$HSC/HPC of donor lost the ability of hematopoietic reconstitution in the recipients gradually. The HSC/HPC derived in the recipients showed lower abilities of self renewing and multi-differentiation, delayed peripheral blood recovery, and resulted in the death of recipients. The HSC/HPS cells displayed characters of aging stem cells: increase of positive rate of SA-β-gal staining, and G_1 cells, lower abilities of self renewing and multi-differentiation, and fewer colonies in the CFU-mix experiment.

Ginseng is one of the important herbs for tonifying qi in traditional Chinese medical science. Ginseng was considered to have the efforts on reinforcing both qi and blood, tranquilizing the mind and promoting intelligence, and prolonging life. TSPG is the main pharmacological composition. Rg1 has the abilities of anti-aging, antioxidation, enhance immunity, but the regulatory mechanism is still not clear [11-13]. Studies showed that Rg1 could prolong the survival time of rat, and could improve the moving ability of aged rat. When cultured in proper concentration Rg1, diploid fibroblasts obtained a higher mitosis activity and faster growth, and the treatment of Rg1 can increase the passage times and prolong cell life [14, 15].

We treated Sca-1$^+$HSC/HPC with Rg1 during the sequential transplantation, results showed that, Rg1 could significantly increase the 30 day survival rate of recipients, and could enhance the Sca-1$^+$HSC/HPC ability of hematopoietic reconstitution in the sequential transplantation. In every generation, the anti-aging group and aging-delay group showed better recovery of WBC, HCT, and PLT than aging group of the same generation. Compared with the aging group, cells in S phase were increased and they showed an enhanced ability of forming CFU-mix colonies. On the other hand, the positive rate of SA-β-Gal staining decreased, and the changes were more significant in the aging-delay group. These results indicated that Rg1 can enhance the potential of self renew and multi-differentiation; there were fewer aged Sca-1$^+$HSC/HPC in the Rg1 treated groups, which indicates an anti aging effect of Rg1 in vivo, and further research showed that Rg1 had a better effect of aging-delay than anti-aging.

The foundation of cell activities is cell cycle. Cells go though proliferation, differentiation, aging, apoptosis, and death in cell cycle. G_1 phase is a key point for the beginning of cell cycle, and there are certain relationships between cell senescence and the arrest of cell

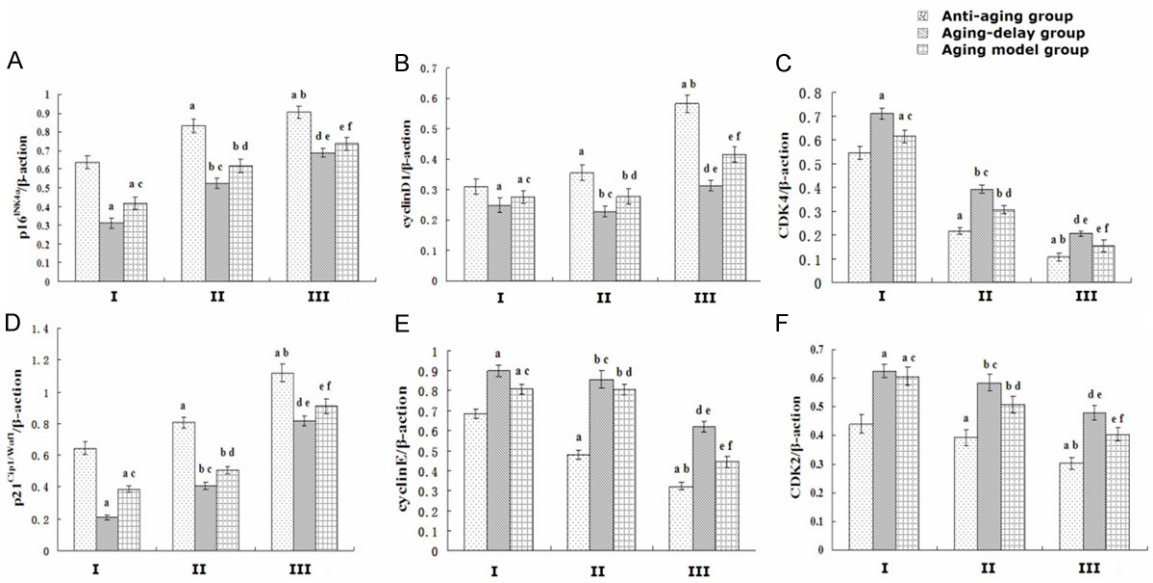

Figure 4. The effect of Rg1 on the expression of Sca-1$^+$HSC/HPC cell cycle regulation protein P16^{INK4a}, CyclinD1, CDK4, P21$^{Cip1/Waf1}$, CyclinE, CDK2. A. The protein expression of P16^{INK4a}, B. The protein expression of cyclinD1, C. The protein expression of CDK4, D. The protein expression of P21$^{Cip1/Waf1}$, E. The protein expression of CyclinE, F. The protein expression of CDK2. I, 1st generation; II, 2nd generation; III, 3rd generation. a. Compared with 1st generation of aging group, $P<0.01$; b. Compared with 2nd generation of aging group, $P<0.05$; c. Compared with 1st generation of anti-aging group, $P<0.05$; d. Compared with 2nd generation of anti-aging group, $P<0.05$; e. Compared with 3rd generation of aging group, $P<0.05$; f. Compared with 3rd generation of anti-aging group, $P<0.05$.

cycle. Aged cells will maintain the metabolic activity for a long time, but they may lose the abilities of DNA synthesis and response of mitogen, for arrested in G_1 phase [16-18]. The process of cell aging is through two signaling pathways, p16INK4a-Rb and pl9Arf-p53-p21Cipl are two of the most important ways, the activation of either pathway will induce the senescence of HSC, when crucial factors involved in these pathways (like INK4 family: including pl6, p15, pl8, and pl9 or CIP/KIP family: including p21, p27, p57) are regulated, cells may begin to grow old or bypass the aging program and proliferate, however, there are wide interactions in different levels between the two pathways, and they are both involved in cell aging sometimes [19, 20]. p16^{INK4a} is one of the most important regulator of cell life, when introduced with p16^{INK4}, cells may show a aged phenotype, and p16^{INK4a} knocked-down cells will process a slower senescence, and have better biological activities, that means the accumulation of p16^{INK4a} is the cause but not the result of senescence. p16^{INK4a} can block the binding of CDK4/6 and CyclinD1 to inhibit the activation of key gene to stop cells from enter S phase, and arrest cells in G_1 phase [21-23]. One of the overlapping genes of INK4a, p19^{Arf} can inhibit the activity of MDM2, and enhance the stability of p53 to induce the expression of p21$^{Cip1/Waf1}$, that will inhibit the CDK4/6-CyclinD1 phosphorylation of Rb and inhibit the activity of CDK2-CyclinE complex, arresting cells in G_1, and as a result, induce cell senescence [24, 25].

In our study, we found that as the transplantation increased, the expression of Sca-1$^+$HSC/HPC p16^{INK4a}, p19^{Arf}, p53, p21$^{Cip1/Waf1}$ mRNA, and the P16^{INK4a}, P21$^{Cip1/Waf1}$, cyclinD1 protein was upregulated, and the expression of CDK4, CDK2, CyclinE was dowregulated, which were in accord with the changes of cell cycle regulators in the cell senescence, indicating that the senescence of Sca-1$^+$HSC/HPC was related with the change of cell cycle regulators. We treated the aging model of Sca-1$^+$HSC/HPC sequential transplantation with Rg1, and detect the changes of cell cycle regulators. Our results showed that, Rg1 can downregulate the expression of p16^{INK4a}, p19^{Arf}, p53, p21$^{Cip1/Waf1}$ mRNA and P16^{INK4a}, P21$^{Cip1/Waf1}$ protein, decrease the inhibition to CDK4, CDK2, increase the expression of CDK4, CDK2, CyclinE protein. Downregulation of p16^{INK4a} can weaken the inhibition

of the activity of cyclinD-CDK complex, decrease the arrest of Sca-1^{+}HSC/HPC in G_1 phase, then negative feedback regulate the synthesis of cyclinD1. After the treatment of Rg1, the expression of cyclinD1 in Sca-1^{+}HSC/HPC was downregulated, indicating that the mechanism of the anti-aging effect on Sca-1^{+}HSC/HPC in sequential transplantation of Rg1 may involve the regulation of cell cycle regulator expression. We speculate that p16^{INK4a}-Rb pathway and p19^{Arf}-p53-p21$^{Cip1/Waf1}$ pathway may play important roles in the Rg1 anti-aging effect. In future studies, we will further research the mechanisms of Rg1 anti-aging effect.

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Disclosure of conflict of interest

None.

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