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Gene expression of the p16^{INK4a}-Rb and p19^{Arf}-p53-p21$^{Cip/Waf1}$ signaling pathways in the regulation of hematopoietic stem cell aging by ginsenoside Rg1

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ABSTRACT. The elucidation of the molecular mechanisms underlying the effects of traditional Chinese medicines in clinical practice is a key step toward their worldwide application, and this topic is currently a subject of intense research interest. Rg1, a component of ginsenoside, has recently been shown to perform several pharmacological functions; however, the underlying mechanisms of these effects remain unclear. In the present study, we investigated whether Rg1 has an anti-senescence effect on hematopoietic stem cells (HSCs) and the possible molecular mechanisms driving any effects. The results showed that Rg1 could effectively delay *tert*-butyl hydroperoxide (t-BHP)-induced senescence and inhibit gene expression in the p16^{INK4a}-Rb and p19^{Arf}-p53-p21$^{Cip/Waf1}$ signaling pathways in HSCs. Our study suggested that these two signaling pathways might be potential targets for elucidating the

molecular mechanisms of the Rg1 anti-senescence effect.

Key words: Hematopoietic stem cells; Rg1; $p16^{INK4a}$; $p19^{Arf}$; Senescence

INTRODUCTION

A traditional Chinese medicine (TCM) compound, salvia pellet, which has been widely used in China for many years, passed phase II of the US Food and Drug Administration (FDA) clinical trial in early 2010, which represents a milestone for the application of TCMs in clinical practice. The worldwide use of TCM has long faced several challenges, including the following: i) it is hard to extract and purify single active ingredients from herb complexes; ii) systematic investigations of side effects are lacking; iii) precise investigations of the molecular mechanisms underlying the TCM effects are needed. However, of all of these challenges, elucidating the molecular mechanisms underlying the effects is the most urgent and important.

Ginseng is a perennial herb belonging to the Araliaceae family and the *Panax* species, which has great medical value and is widely used in Asia. In recent decades, ginseng was also introduced to western countries where it became a popular medicine (Helms, 2004). Ginseng was reported to have several pharmacological functions (Buettner et al., 2006; Sun et al., 2012), and isolated and purified forms of the major components and effective functional molecule in ginseng root, ginsenosides, are now attracting increasing research interest (Cheng et al., 2005). Of particular current interest are investigations aimed at determining the detailed mechanisms underlying the action of ginsenosides, which would help to clarify the discrepancies found in previous literature. Ginsenosides appear to be the active component for most of ginseng's pharmacological effects, including its vasorelaxation, anti-oxidation, anti-inflammation, and anti-cancer properties. Based on differences in molecular structure, there are almost 40 ginsenoside isoforms, and the most commonly studied are Rb1, Rg1, Rg3, Re, and Rd. Their effects on the central and peripheral nervous system have been reviewed (Nah et al., 2007), and the anti-amnestic and anti-aging effects of Rb1 and Rg1 were also previously studied (Cheng et al., 2005).

Hematopoietic stem cells (HSCs) are multipotent stem cells in the blood cell lineage that can differentiate into all kinds of mature blood cells to maintain blood cell homeostasis. In the process of cell development and differentiation, the movement through cell cycle checkpoints is regulated by several types of molecules, of which members of the cyclin-dependent kinase (CDK) family are especially important (Morgan, 1995).

CDK activation requires binding with cyclins and many phosphorylation processes (Solomon and Kaldis, 1998). Passing through the G1/S checkpoint requires the phosphorylation of Rb, which is primarily mediated by cyclinD1-associated CDK4 or CDK6, as well as cyclinE-associated CDK2 (Dowdy et al., 1993). Two families of CDK inhibitors, INK4 and KIP, are also involved in regulating CDK activity (Sherr and Roberts, 1999). The KIP family contains three members, $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$, which can all bind to the cyclin-CDK complex and thus inhibit its activity. Recent *in vitro* experiments demonstrated that a single KIP molecule was sufficient to inhibit cyclin/CDK activity. In contrast to the KIP inhibitors, members of the INK4 family ($p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, and $p19^{INK4D}$) can specifically bind to CDK4 and CDK6, and consequently reduce their binding potential to cyclinD to ultimately inhibit cyclin/CDK activity.

By using *in vitro* cell models, a role of CDK inhibitors in cell senescence was identified. An increasing expression level of p21 and/or p16 has been found in aged human and murine fibroblasts and melanocytes (Zindy et al., 1997). The elevated expression of p16 and p21 in a cell senescence model was found to be associated with the degree of inhibition of CDKs at the G1-S phase. Elimination of *p21* gene expression through homologous recombination extended the lifespan of human diploid fibroblasts *in vitro* (Brown et al., 1997). Thus, the loss of *p21*, although not sufficient to abrogate senescence, may represent a key factor in the fibroblast lifespan. Furthermore, data from a knockdown experiment of the *p21* and *p16* genes in human cancer tissues *in vivo* also suggested that these inhibitors may contribute to the senescence checkpoint and ultimately influence tumor development (Tsihlias et al., 1999).

Although some recent reports suggested that ginsenoside Rg1 had anti-aging effects, the underlying molecular mechanisms remain to be clarified (Chen et al., 2008). Few reports have focused on how Rg1 regulates HSC senescence. We previously successfully established an *in vitro tert*-butyl hydroperoxide (t-BHP)-induced mouse Sca-1+ HSCs senescence cell model (Zhou et al., 2011). In the present study, we employed our *in vitro* cell senescence model to investigate the effect of Rg1 on senescence in HSCs. Moreover, the gene expressions of several key signal transduction molecules in the $p16^{INK4a}$-Rb and $p19^{Arf}$-p53-$p21^{Cip/Waf1}$ pathways were also investigated.

MATERIAL AND METHODS

Cell culture and Rg1 treatment

The Sca-1+ HSCs senescence cell model was established as previously described (Zhou et al., 2011). HSCs were harvested by flushing the tibia and femoral content of 6-8-week-old C57BL/6 mice weighing 20-25 g (Chongqing Medical and Experimental Animal Center) with buffer containing 2 mM ethyylenediaminetetraacetic acid (EDTA) and 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.2. The mixture was filtered through a 30-μm filter membrane and marrow nucleated cells (MNCs) were isolated with Ficoll separating buffer. Approximately 5-8 x 10^7 MNCs were incubated with 90 μL buffer and 10 μL anti-Sca-1-fluorescein isothiocyanate (FITC) (Miltenyi) at 4°C for 10 min. The cell mixture was centrifuged, the cell pellet was collected, and 80 μL buffer and 20 μL anti-FITC microbeads were added. The samples were incubated at 4°C for 20 min, centrifuged again, and the cell pellet was collected and resuspended in 0.5 mL buffer. Next, a magnetic column was used to isolate Sca-1+ cells, followed by flow cytometry analysis and Trypan Blue staining to verify the cell purity and survival rate.

Sca-1+ HSCs were cultured on a 6-well plate at a 1 x 10^5 cells/well density and divided into 5 groups: i) blank group: HSCs were incubated in Iscove's modified Dulbecco's medium (IMDM)/10% fetal bovine serum (FBS) (Gibco) at 37°C and 5% CO_2 for 12 h; ii) t-BHP (Sigma) group: HSCs incubated in 100 μM t-BHP for 6 h and then in IMDM for a further 6 h; iii) Rg1 (Jilin Hongjiu Biological Science and Technology Ltd.; purity ≥95%; Figure 1) group: HSCs were incubated in 10 μM Rg1 for 12 h; iv) t-BHP + Rg1 group: HSCs were incubated in 100 μM t-BHP for 6 h and then in 10 μM Rg1 for 6 h; v) Rg1 + t-BHP group: HSCs were incubated in 10 μM Rg1 for 6 h and then in 100 μM t-BHP for 6 h.

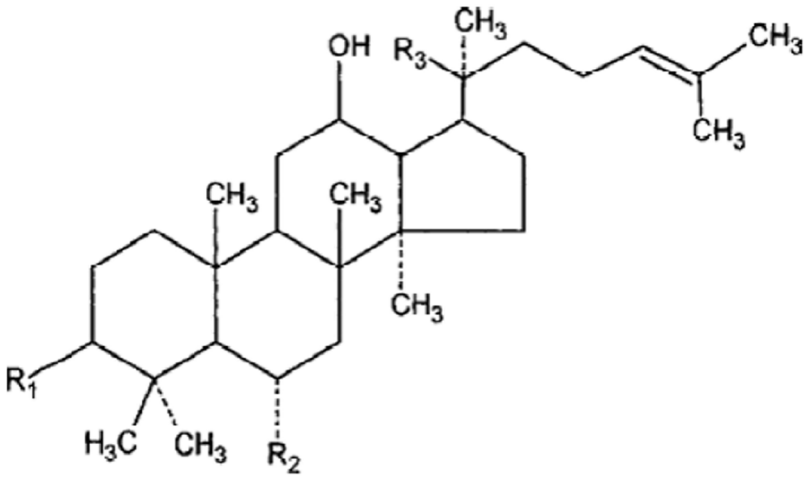

Figure 1. Structure of ginsenoside Rg1. R_1 = OH; R_2 = OGlc; R_3 = OGlc.

Senescence-associated beta galactosidase (SA-β-gal) staining

The HSCs were collected and washed twice with PBS. Then, the cells were stained with staining buffer (Cell Signaling Technology) at 37°C for 12 h. Four hundred cells per slide were randomly counted and the positive staining rate was calculated.

Flow cytometry analysis

The HSCs were collected and washed with PBS. The cells were then fixed with 70% ice-cold alcohol overnight, washed twice more in PBS, and then incubated in 1 mg/mL ribonuclease at 37°C for 30 min. Then, 50 μg/mL propidium iodide (PI) was added and cells were incubated for another 30 min. Flow cytometry was employed to investigate the cell cycle.

Colony forming unit (CFU)-Mix assay

The effect of Rg1 on cell senescence was evaluated by adding 1 x 10^{-4} M 2-mercaptoethanol, 3% L-glutamine (Sigma), horse serum (Gibco), erythropoietin (EPO) (R&D), interleukin (IL)-3, granulocyte macrophage-colony stimulating factor (GM-CSF) (R&D), and 2.7% methylcellulose (Sigma) to 1 x 10^4 HSCs on a 96-well plate. Cells were incubated at 37°C and 5% CO_2 for 7 days and the number of CFUs were counted.

Real-time polymerase chain reaction (PCR)

Total RNA was isolated using TRIzol reagent (Takara) according to manufacturer instructions. After the reverse transcription reaction, real-time PCR was performed in an ABI 7900HT system using SYBR®Premix Ex Taq™ (Bio-Rad) according to manufacturer instructions. The conditions of real-time PCR were as follows: 40 cycles at 94°C for 10 s and 60°C

for 30 s. A dissociation stage was added to the end of the amplification procedure. There was no non-specific amplification determined by the dissolve curve. β-actin was used as the internal control. The primer sequences are listed in Table 1.

Table 1. Primer sequences in real-time PCR.

Gene	Primer sequence (5'-3')	Product size (bp)
$P16^{INK4a}$	F: 5'-ATGGAGTCCFCTGCAGACAG-3' R: 5'-ATCGGGGTACGACCGAAAG-3'	121
$P19^{Arf}$	F: 5'-GGGTCGCAGGTTCTTGGTC-3' R: 5'-GTGCGGCCCTCTTCTCAA-3'	172
P53	F: 5'-CCCCAGGATGTTGAGGAGTT-3' R: 5'-TTGAGAAGGGACAAAAGATGACA-3'	153
$P21^{Cip1/Waf1}$	F: 5'-ACTTCCTCTGCCCTGCTGC-3' R: 5'-GGTCTGCCTCCGTTTTCG-3'	138
β-ACTIN	F: 5'-GAGACCTTCAACACCCCAGC-3' R: 5'-ATGTCACGCACGATTTCCC-3'	263

Western blot

Cells were lysed on ice for 30 min in lysis buffer (Applygen), containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.1% sodium dodecyl sulfate (SDS) supplemented with protease inhibitors (10 g/mL leupeptin, 10 g/mL pepstatin A, and 10 g/mL aprotinin). Proteins were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to a nitrocellulose membrane, and detected using anti-$p16^{INK4a}$, -CDK4, -cyclinD, -$p21^{Cip1/Waf1}$, -CDK2, and -β-actin (Santa Cruz) antibodies and appropriate secondary antibodies. Enhanced chemiluminescence solution (Pierce) was used for development.

Statistical analysis

Statistical significance was calculated by using a factorial design and one-way analysis of variance (ANOVA) in the SPSS 16.0 software. The Tukey test was used to evaluate significant differences revealed in the ANOVA. Statistical significance was analyzed in data from at least three independent experiments. P values <0.05 were considered to be statistically significant. All data are reported as means ± SD unless labeled otherwise.

RESULTS

HSC senescence is regulated by Rg1

To investigate the effect of ginsenoside Rg1 on HSC senescence, we first established a cell model of HSC senescence induced by t-BHP. An SA-β-gal staining assay was employed to confirm whether the *in vitro* senescence model was successfully established. Results showed that compared with the control group, there were more blue positively stained cells after t-BHP treatment (Figure 2). We next administered Rg1 and t-BHP to HSCs both separately and together, and counted the number of positively stained HSCs, which reflected the degree of cell senescence. Results showed that Rg1 could effectively reverse the t-BHP-induced HSC senescence, regardless of whether it was added to cells before or after t-BHP; however, pre-treatment of HSCs with Rg1 before adding t-BHP had a more remarkable effect (Figure 3A).

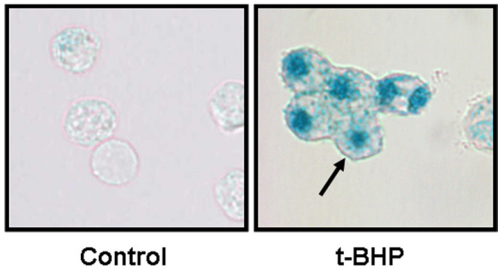

Figure 2. SA-β-gal staining of hematopoietic stem cells in response to t-BHP treatment. Arrow indicates positive staining.

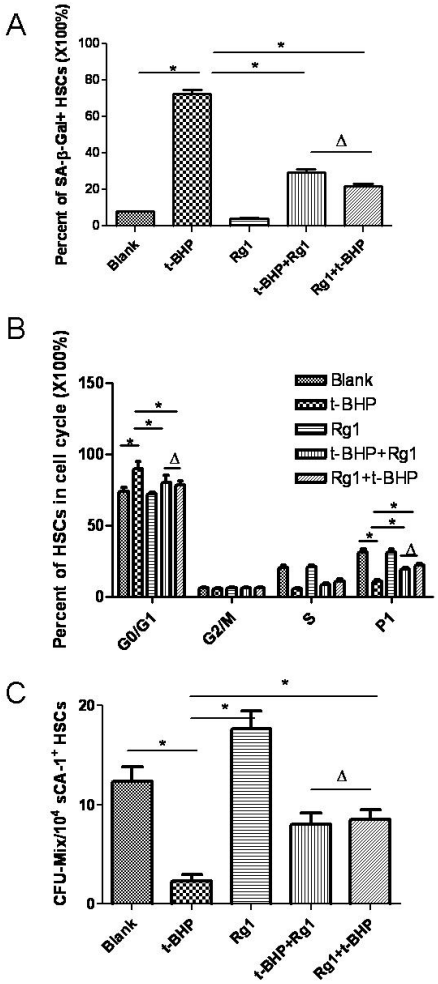

Figure 3. Hematopoietic stem cells (HSC) senescence in response to t-BHP with or without Rg1. SA-β-gal staining (**A**), cell cycle analysis by flow cytometry (**B**), and CFU-mix assay (**C**) in each group, from left to right: blank control, t-BHP, Rg1, t-BHP+Rg1, and Rg1 + tBHP, analyzed separately, were employed to study the effect of Rg1 on HSC senescence. Data are reported as means ± SD. $^{\Delta}P < 0.05$, $^{*}P < 0.01$.

The flow cytometry assay was then performed to detect changes in the HSC cell cycle in response to t-BHP and Rg1 treatments. As shown in Figure 3B, the percentage of HSCs in the G_0/G_1 phase was significantly enhanced in the t-BHP group, which indicated that t-BHP treatment induced G_1 phase arrest, whereas the combined treatment with Rg1 could relieve the extent of G_1 phase arrest induced by t-BHP. Furthermore, the G_2/M and S phase percentages and the proliferation index all showed a similar pattern as that of the G_0/G_1 phase results (Figure 3B).

Finally, the CFU-Mix assay was performed to evaluate the effect of Rg1 on t-BHP-induced HSC senescence. Results showed that compared with the control, the number of HSC colonies was significantly reduced in the t-BHP group, whereas the combined treatment with Rg1 could prevent the decrease in colony number. Furthermore, Rg1 treatment alone could also increase the colony number (Figure 3C).

Expression of $p16^{INK4a}$-Rb and $p19^{Arf}$-p53-$p21^{Cip/Waf1}$ signaling pathway genes in the regulation of HSC senescence by Rg1

Having observed that Rg1 could effectively reverse t-BHP-induced HSC senescence, we next investigated the molecular mechanisms underlying this effect. For this purpose, we detected the mRNA expression level of several signaling molecules, including $p16^{INK4a}$, $p19^{Arf}$, p53, and $p21^{Cip/Waf1}$, involved in the $p16^{INK4a}$-Rb and $p19^{Arf}$-p53-$p21^{Cip/Waf1}$ signaling pathways by real-time PCR. Results showed that whether added prior to or after t-BHP treatment, Rg1 could effectively reverse the enhancement of $p16^{INK4a}$ mRNA expression in response to t-BHP treatment. Moreover, administration of Rg1 prior to t-BHP had a better effect than when it was administered after (Figure 4A). The other mRNAs, including $p19^{Arf}$, p53, and $p21^{Cip/Waf1}$, all showed similar patterns of change to that of $p16^{INK4a}$ in response to Rg1 and t-BHP treatment (Figure 4B, C, and D).

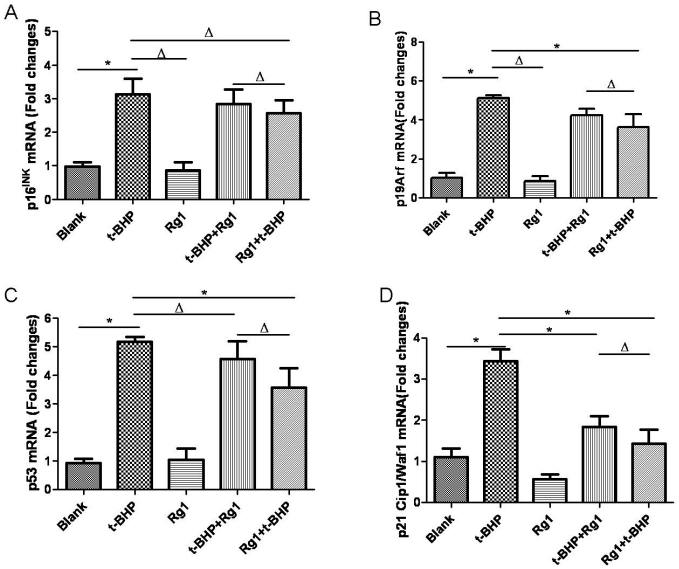

Figure 4. mRNA expression pattern of $p16^{INK4a}$, $p19^{Arf}$, p53, and $p21^{Cip/Waf1}$ in the regulation of HSC senescence by ginsenoside Rg1. Total RNA was extracted from HSCs treated with t-BHP and/or Rg1 and subjected to real-time PCR using primers for $p16^{INK4a}$ (**A**), $p19^{Arf}$ (**B**), p53 (**C**), and $p21^{Cip/Waf1}$ (**D**). β-actin was used as the internal control and the results are expressed as fold changes in mRNA abundance. Data are reported as means ± SD. *$P < 0.01$, $^{\Delta}P < 0.05$.

Finally, we detected the protein expressions in these two signaling pathways. Results showed that in response to t-BHP treatment, $p16^{INK4a}$, cyclinD1, and $p21^{Cip/Waf1}$ were upregulated, whereas CDK4, cyclinE, and CDK2 were downregulated. Furthermore, Rg1 treatment could reverse all of these patterns to some extent, and had a better effect when treated prior to t-BHP administration (Figure 5).

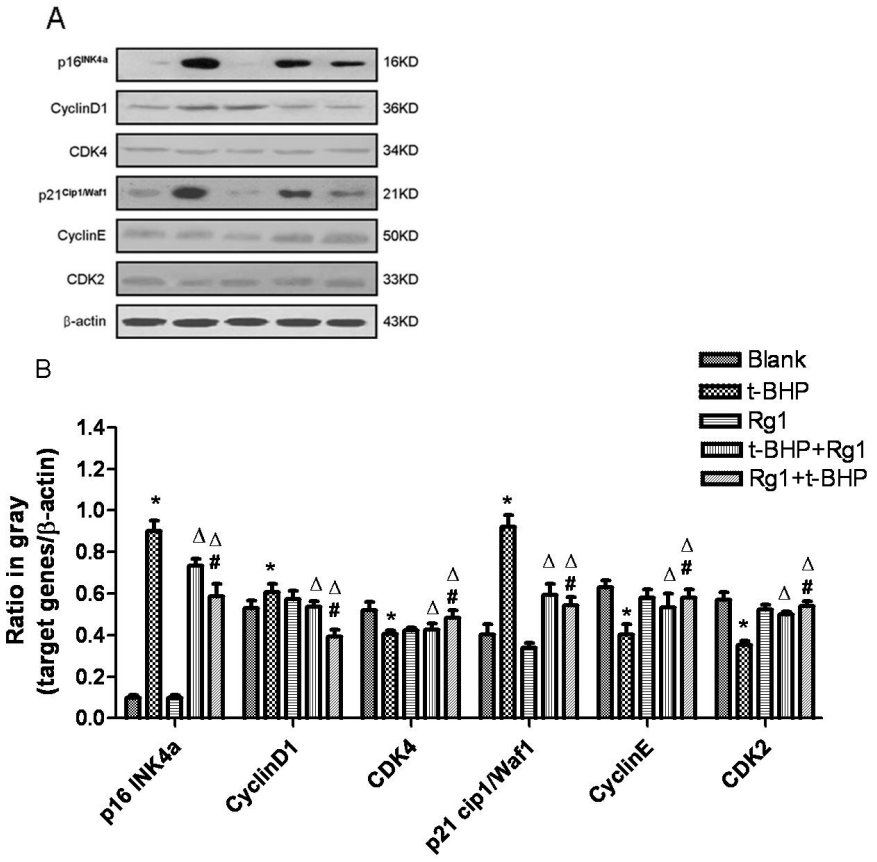

Figure 5. Protein expression pattern of the $p16^{INK4a}$-Rb and $p19^{Arf}$-p53-$p21^{Cip/Waf1}$ signaling pathways in the regulation of HSC senescence by ginsenoside Rg1. Total protein was extracted from HSCs treated with t-BHP and/or Rg1 and subjected to Western blot analysis using anti- $p16^{INK4a}$, -cyclinD1, -CDK4, -$p21^{Cip/Waf1}$, -cyclinE, and -CDK2 antibodies (**A**). β-actin was used as internal control. Band intensity screening was performed to quantify protein expression (**B**). The result was expressed as the ratio of the intensity of target gene expression to β-actin expression in each group, from left to right: blank control, t-BHP, Rg1, t-BHP + Rg1, and Rg1 + tBHP. *$P < 0.01$, t-BHP *vs* blank; $^{\Delta}P < 0.01$, t-BHP + Rg1 and Rg1 + t-BHP *vs* t-BHP; $^{\#}P < 0.05$, Rg1 + t-BHP *vs* t-BHP + Rg1.

DISCUSSION

The regulation of the senescence of stem cells is a new focus of aging research (Rossi et al., 2007). The senescence of stem cells would lead to a gradual loss of their renewal and multi-potential differentiation ability, and could even disturb their proliferation and differ-

entiation, which would result in severe disorders in the structure or function of tissues, and thus facilitate and accelerate disease generation and development. HSC senescence is closely related to the human aging process, inhibiting hematopoiesis, impairing immune system function, and facilitating tumorigenesis including senile leukemia and aplastic anemia. Therefore, the molecular mechanisms underlying HSC senescence and the identification or development of drugs that can delay senescence in HSCs is currently an area receiving more and more research interest.

Ginsenoside is the main pharmaceutical component of ginseng, and the Rg1 isoform shows beneficial effects on the anesis aging condition, including the attenuation of oxidation impairments and improvement of immune system function (Shi et al., 2011; Yu et al., 2012), However, the underlying mechanisms remain unclear. Our previous research suggested that ginsenoside could promote the proliferation of human hematopoietic stem/progenitor cells and induce their differentiation along certain directions. In addition, other studies also demonstrated that Rg1 at certain concentrations could prolong the survival time of aged rats and elongate the lifespan of fibroblasts by increasing cell viability (Cheng et al., 2005; Chen et al., 2008). The results of these studies suggested that Rg1 might function as an anti-aging reagent via increasing cell viability and degeneration.

t-BHP, a reactive hydroperoxide produced from lipid peroxidation, has been shown to be a major molecule involved in the activation of redox-sensitive transcription factors (Lee et al., 2005), and is frequently used in investigations of free radical-induced cellular alterations. Given its high hydrophobicity, t-BHP critically affects biological membranes. Low concentrations of t-BHP could induce the peroxidation of cellular lipids at levels that are causally related to cell death. t-BHP also inhibits glutathione peroxidase, which detoxifies lipid hydroperoxides, and induces damage to proteins and DNA. Furthermore, sublethal oxidative stresses induced by a low amount of t-BHP were shown to induce a shift from early fibroblast morphotypes to the later forms. Observations based on a time-lapse video of single cells after stress stimulation clearly showed a morphotype transition from one stage to the next (Toussaint et al., 2000). Therefore, t-BHP is now widely used in research related to senescence both *in vivo* and *in vitro* (Maurya and Rizvi, 2009). In the current study, consistent with previous results (Rossi et al., 2007), we also found that after t-BHP treatment, HSCs showed proliferation inhibition, G_1 phase arrest, remarkable SA-β-gal positive staining, and decreased CFU-mix and multi-potential, which indicated that our model was successful.

The cell cycle and senescence are closely linked through some specific signaling pathways, and it is known that the $p16^{INK4a}$-Rb and $p19^{Arf}$-MDM2-p53-$p21^{Cip1/Waf1}$ signaling pathways are involved in cell senescence (Noda et al., 2009). Janzen et al. (2006) demonstrated that young cells showed the senescence phenotype through overexpression of $p16^{INK4a}$, and senescence was delayed in a knockout $p16^{INK4a}$ HSC neural stem cell line, which suggested that $p16^{INK4a}$ was a key gene in the regulation of the lifespan of certain cells. $p16^{INK4a}$ could block CDK4/6-induced Rb phosphorylation through inhibition of CDK4/6 binding to cyclinD1, which could in turn inhibit E2F release, suppress the expression of many S-phase-related genes, and finally lead to G_1 phase arrest. $p19^{Arf}$ could directly bind to MDM2 and inhibit the degradation of p53 function, and thus enhance the stability and activity of p53 (Merkel et al., 2010). $p21^{Cip1/Waf1}$, a response gene to p53, could be induced by p53 and then inhibit the CDK4/6-cyclinD1 complex-induced Rb phosphorylation and activity of the CDK2/cyclinE complex, which could finally result in G_1 phase arrest and cell senescence (van Os et al., 2007; Yu et al., 2010).

In the present study, we established an *in vitro* HSC senescence cell model that was induced by administration of t-BHP. In the aging cell group, we found that both the mRNA and protein expression levels of $p16^{Ink4a}$ increased and that the expression of the downstream protein cyclinD1 also increased, whereas the expression of CDK4 decreased. Furthermore, the $p16^{INK4a}$, $p19^{Arf}$, p53, and $p21^{Cip1/Waf1}$ mRNA expression levels and the $P21^{Cip1/Waf1}$ protein expression level increased, whereas the CDK2 and cyclinE expression levels decreased. These results coincided with molecular changes observed in cells under the aging condition. Furthermore, the results showed that after Rg1 treatment, the $p16^{Ink4a}$ mRNA and protein expression levels decreased and the inhibition of CDK4/6-cyclinD1 was prevented, which indicated that Rg1 might delay Sca-1+ HSC aging through the $P16^{Ink4a}$-Rb pathway. On the other hand, after Rg1 treatment, the $p19^{Arf}$, p53, and $p21^{Cip1/Waf1}$ mRNA levels and the $P21^{Cip1/Waf1}$ protein expression level decreased, whereas the CDK2 and cyclinE expression levels increased. These results indicated that Rg1 could also have some effects on the $p19^{Arf\text{-}Mdm2}$-p53-$p21^{Cip1/Waf1}$ pathway. Moreover, the effect of Rg1 pre-treatment was more significant than that after t-BHP induction. Therefore, this study provides evidence that these two signaling pathways might be potential targets for elucidating the molecular mechanisms underlying the Rg1 anti-senescence effect.

CONCLUSION

Rg1 could delay t-BHP-induced HSC senescence *in vitro* and reverse the expression patterns of the genes involved in the $p16^{INK4a}$-Rb and $p19^{Arf}$-p53-$p21^{Cip/Waf1}$ signaling pathways during t-BHP-induced HSC senescence. Taken together, the results suggested that these two signaling pathways might be potential targets for elucidating the molecular mechanisms underlying the Rg1 anti-senescence effect.

Conflicts of interest

The authors declare no conflict of interest.

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