

Epigallocatechin-3-gallate prevents oxidative stress-induced cellular senescence in human mesenchymal stem cells via Nrf2

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Abstract. Human mesenchymal stem cells (hMSCs) have great therapeutic potential due to their high plasticity, immune privileged status and ease of preparation, as well as a lack of ethical barriers to their use. However, their ultimate usefulness is limited by cellular senescence occurring secondary to increased cellular levels of reactive oxygen species (ROS) during their propagation in culture. The underlying molecular mechanisms responsible for this process in hMSCs remain unclear. An antioxidant polyphenol epigallocatechin-3-gallate (EGCG) found in green tea, is known to activate nuclear factor-erythroid 2-related factor 2 (Nrf2), a master transcriptional regulator of antioxidant genes. Herein, we examined the EGCG-mediated antioxidant mechanism in hMSCs exposed to ROS which involves Nrf2 activation. The H₂O₂-exposed hMSCs showed cellular senescence with significantly increased protein levels of acetyl-p53 and p21 in comparison with the untreated hMSCs, and these effects were prevented by pre-treatment with EGCG. By contrast, in Nrf2-knockdown hMSCs, EGCG lost its antioxidant effect, exhibiting high levels of acetyl-p53 and p21 following EGCG pre-treatment and H₂O₂ exposure. This indicates that Nrf2 and p53/p21 may be involved in the anti-senescent effect of EGCG in hMSCs. Taken together, these findings indicate the important role of EGCG in preventing oxidative stress-induced cellular senescence in hMSCs through Nrf2 activation, which has applications for the massive production of more suitable hMSCs for cell-based therapy.

Introduction

Bone marrow-derived human mesenchymal stem cells (hMSCs) are a desirable cell source for cell-based therapy

owing to their high plasticity, immune privileged status and ease of preparation, as well as a lack of ethical barriers to their use. They also have high self-renewal capacity with sustained proliferation *in vitro* (1,2). However, obtaining the large numbers of cells required for therapeutic applications is often problematic as hMSCs are subject to the Hayflick limit, a finite proliferation capacity *in vitro* and replicative senescence after long-term culture (3-5). Senescent cells have shown reduced multipotency, clonogenicity and subsequent arrest of proliferation, thus limiting the regenerative potential of hMSCs necessary for the desired therapeutic effects (5).

Cellular senescence is characterized by irreversible cell cycle arrest, despite continued metabolic activity and viability. Senescence is caused by inadequate culture conditions, such as culture shock or cellular stress (3,4). The stress-induced premature senescence (SIPS) of human stem cells may be induced by subcytotoxic stress (H₂O₂, histone deacetylase inhibitors and radiation) (5,6).

Oxidative stress, mediated by reactive oxygen species (ROS) including hydrogen peroxide (H₂O₂), superoxide anion radical, hydroxyl radical and peroxide, plays a crucial role in the induction of SIPS (3,4). Sublethal concentrations of H₂O₂ may damage cellular components including DNA, which leads to low metabolic activity and cell cycle arrest through the activation of either the p53/p21 or the p16/pRb pathway (7). Notably, p53 acetylation, which is induced by Sirt1, the human homolog of yeast SIR2, has been proposed to promote senescence (8-11). Acetylation of p53 is a translational modification that results in the activation of p53. Cellular senescence was observed in serially-passaged and H₂O₂-treated human dermal fibroblast cells and acetyl-p53 levels were markedly increased compared with phosphorylated p53 levels (12). These findings suggest an association between oxidative stress-mediated senescence and p53 acetylation.

Polyphenols, or polyphenolic compounds, are widely distributed in nature. Polyphenols, such as the green tea polyphenol epigallocatechin-3-gallate (EGCG), have been demonstrated to exhibit various biological properties, including DNA damage protection and free radical scavenging (13). Furthermore, polyphenols are pharmacologically safe compounds in humans (14). In addition to the ability to act as a neutralizing agent of excessive ROS, EGCG exerts antioxidant, anti-inflammatory and anti-tumorigenic effects (15). Recently, EGCG has been shown to suppress H₂O₂-mediated apoptotic cell death in hMSCs (16). It is well

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known that EGCG exerts an antioxidant effect by activating the nuclear factor-erythroid 2-related factor 2 (Nrf2) signaling pathway, which is involved in the cellular antioxidant defense system (17). Nrf2 activation is closely regulated by Kelch-like ECH-associated protein 1 (Keap1), a substrate adaptor for Cul3-based E3 ligase, which targets Nrf2 for proteasomal degradation (18). In response to oxidative stress, Nrf2 upregulates the expression of antioxidant and detoxifying genes by binding to antioxidant response elements (AREs) in the promoter region of the encoding genes (19,20).

The purpose of this study was to examine the novel molecular mechanisms underlying the anti-senescent effect of EGCG in H₂O₂-exposed hMSCs. Our data demonstrated that EGCG reversed H₂O₂-induced oxidative stress by downregulating the p53-p21 signaling pathway and upregulating Nrf2 expression. Nrf2-knockdown hMSCs showed significantly increased protein levels of acetyl-p53 and p21 following EGCG pre-treatment and H₂O₂ exposure, which suggests a potential role for Nrf2 in p53/p21 regulation to thereby prevent oxidative stress-induced cellular senescence in hMSCs.

Materials and methods

Culture of hMSCs. Adult bone marrow-derived hMSCs were purchased from Cambrex (Walkersville, MD, USA). hMSCs (passages 4-10) were cultured in Dulbecco's modified Eagle's medium (DMEM) low glucose containing 10% fetal bovine serum (FBS) (both from Gibco, Grand Island, NY, USA) at 37°C with 5% CO₂.

EGCG treatment and exposure of cells to H₂O₂. EGCG and H₂O₂ were purchased from Sigma-Aldrich (St. Louis, MO, USA). To define the optimal concentrations for use in subsequent experiments, hMSCs were pre-incubated with different amounts of EGCG (50 and 100 μM) for 6 h and then the cells were exposed to 200 μM H₂O₂ (diluted in DMEM supplemented with 10% FBS) for 2 h. The cells were washed twice with DMEM to remove excess H₂O₂ and re-incubated in fresh complete medium for 24 h to prevent cell death and allow for the observation of senescent characteristics

Cellular senescence assay. The activity of senescence-associated β-galactosidase (SAβ-gal), a marker of senescence, was analyzed in hMSCs using a cellular senescence assay kit (EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Briefly, the medium was aspirated and the cells were washed once with phosphate-buffered saline (PBS; pH 6.0). After fixing the cells with 1X fixing solution at room temperature for 10 min, the cells were washed again with PBS and incubated without light for at least 4 h with prepared SAβ-gal detection solution at 37°C without CO₂. The percentage of senescence-stained cells was obtained by counting the number of blue-stained cells and the total number of cells per field under the microscope (CKX41; Olympus, Tokyo, Japan; 100-200 cells in four random fields).

Cell viability assay. Cell viability was analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Briefly, hMSCs were cultured in 24-well tissue culture plates and exposed to 200 μM H₂O₂ for

2 h. After 24 h, the cells were stained with 1 mg/ml MTT (Sigma-Aldrich). The media were then carefully aspirated and 150 μl dimethyl sulfoxide (DMSO) was added to solubilize the colored formazan product. The optical density was read at 554 nm using a microplate reader (Floustar Optima; BMG Labtech, Ortenberg, Germany).

Western blot analysis. The cells were washed twice with cold PBS and lysed with RIPA buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.2 mg/ml leupeptin, 0.2 mg/ml aprotinin, 0.1 M phenylmethylsulfonylfluoride (PMSF), 1 mM Na₃VO₄ and 0.5 M NaF]. The lysates were centrifuged at 13,500 x g for 15 min at 4°C and the supernatants were loaded on to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The following primary antibodies were used: rabbit anti-p53 (1:1,000; sc-6243; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); rabbit anti-acetyl p53 (1:1,000; 06-758; Upstate Biotechnology, Lake Placid, NY, USA); mouse anti-p21 (1:2,000; sc-6246) and rabbit anti-Nrf2 (1:1,000; SC-722) (both from Santa Cruz Biotechnology, Inc.); mouse anti-α-tubulin (1:5,000; T5168; Sigma-Aldrich) and goat anti-lamin B (1:2,000; sc-6216; Santa Cruz Biotechnology, Inc.). Primary antibodies were detected using horseradish peroxidase-conjugated goat anti-mouse (A2554), -rabbit (A0545) (Sigma-Aldrich), or donkey anti-goat secondary antibodies (sc-2020; Santa Cruz Biotechnology, Inc.) and visualized using an enhanced chemiluminescence detection system (Thermo Fisher Scientific, Rockford, IL, USA).

Subcellular fractionation. To obtain nuclear and cytoplasmic fractions, the cells were harvested and suspended in ice-cold cytoplasmic lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT and 0.2 mM PMSF) on ice for 15 min. The suspensions were then centrifuged at 13,500 x g for 10 min at 4°C and the supernatants were saved as the cytoplasmic fractions. The pellets were resuspended in nuclear lysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 0.4 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF) and incubated on ice for 40 min with occasional gentle shaking. The suspensions were then centrifuged at 13,500 x g for 15 min and the supernatants were used as nuclear fractions. Quantification of the results of western blot analysis was performed using ImageJ software (NIH, Bethesda, MD, USA).

Immunocytochemistry. The hMSCs were pre-incubated with 100 μM EGCG for 6 h, fixed in PBS containing 4% PFA and incubated overnight at 4°C with rabbit anti-Nrf2 (1:100). Alexa Fluor 546 anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) was used as a secondary antibody. The cells were counterstained with 100 ng/ml 4,6-diamidino-2-phenylindole (DAPI) (Santa Cruz Biotechnology, Inc.) for nuclear staining and visualized using a confocal laser scanning microscope (FV300; Olympus).

Transfection of small interfering RNA (siRNA). Human Nrf2-specific siRNA oligonucleotides (SMARTpool) were purchased from Dharmacon (Lafayette, CO, USA). The following target specific siRNA sequences were used: 5'-UAAAGUGGCUCAGAAU-3'; 5'-GAGUACAGUG

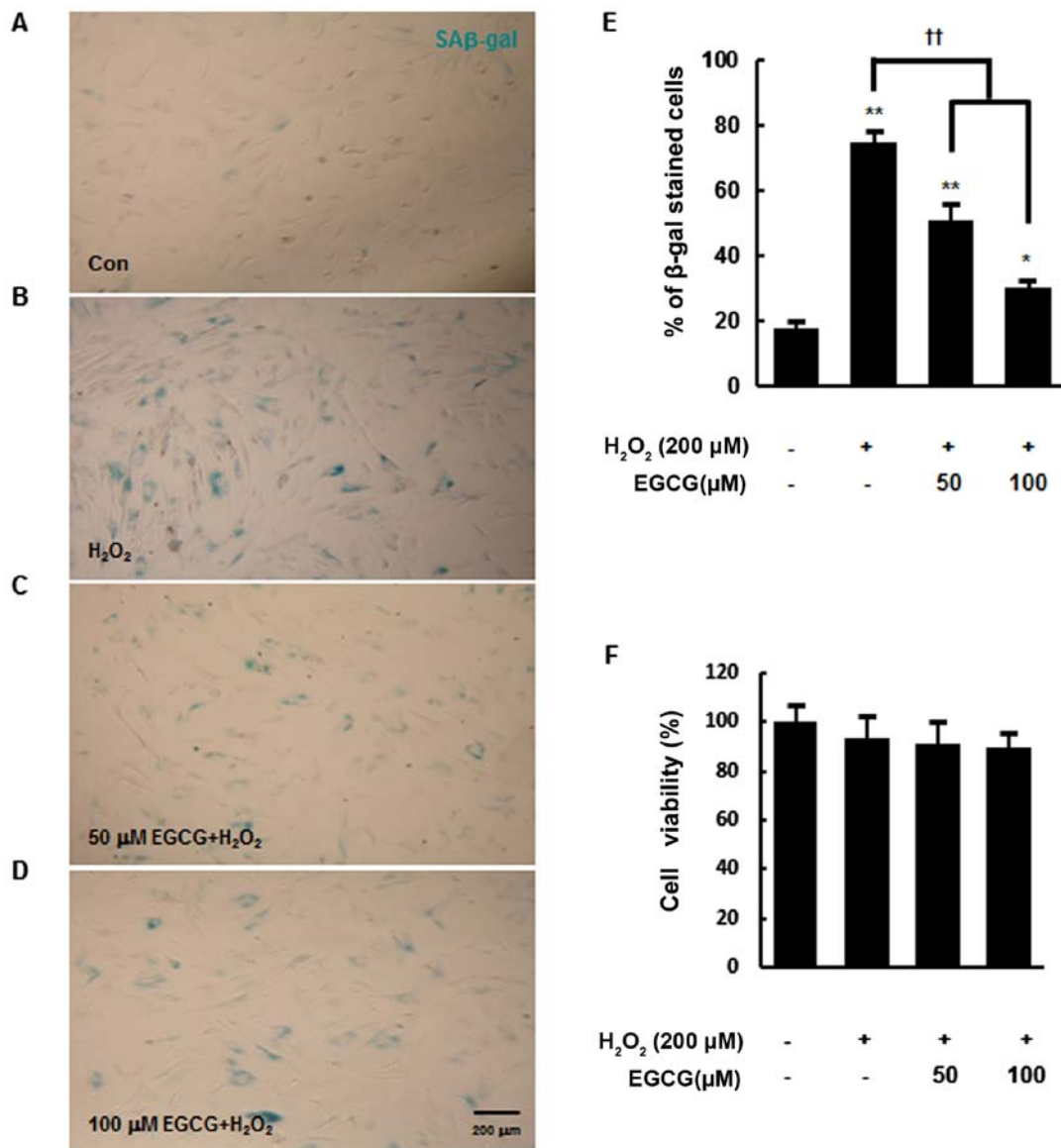


Figure 1. Epigallocatechin-3-gallate (EGCG) pre-treatment reduces cellular senescence in H₂O₂-treated human mesenchymal stem cells (hMSCs). (A-D) Senescence-associated β-galactosidase (SAβ-gal) staining of control (Con) and hMSCs before and after H₂O₂ exposure. hMSCs were treated with 50 or 100 μM of EGCG for 6 h and then exposed to H₂O₂ (200 μM) for 2 h. Twenty-four hours after H₂O₂ exposure, the cells were stained with SAβ-gal (blue cytoplasmic stain). Scale bar, 200 μm. (E) Quantification of SAβ-gal activity. (F) Cell viability of hMSCs. MTT assays were performed 24 h after H₂O₂ exposure. Changes in cell survival observed in three independent experiments are presented as the means ± SEM. *P<0.05 and **P<0.01 vs. the control; ††P<0.01 vs. the H₂O₂ groups. ANOVA followed by a post hoc Newman-Keuls test.

UCUAAUA-3'; 5'-UGGAGUAAGUCGAGAAGUA-3'; and 5'-CACCUAAUAUCUCGAAGUU-3'. Non-targeting scrambled 20-25 nt siRNA oligonucleotides (Santa Cruz Biotechnology, Inc.) were used as a control. Transient transfections were performed using DharmaFECT 3 transfection reagent (Dharmacon) according to the manufacturer's instructions. Briefly, siRNA/lipid complexes were added to the wells at a final concentration of 100 nM siRNA and 1 μl/well of DharmaFECT 3. Nrf2 gene expression was determined at 48 h after transfection.

Statistical analysis. Statistical analysis was performed by analysis of variance (ANOVA) followed by a post hoc Newman-Keuls test. P-values <0.05 were considered to indicate a statistically significant difference. All data are presented as the means ± standard error of mean (SEM).

Results

EGCG pre-treatment reduces cellular senescence in H₂O₂-treated hMSCs. The stimulation of cells with exogenous ROS activates various signaling pathways that result in DNA damage, cellular senescence and apoptosis (3). In order to examine the effects of H₂O₂ exposure on cellular senescence, the hMSCs were exposed to 200 μM H₂O₂ diluted in DMEM supplemented with 10% FBS for 2 h, in order to allow the observation of senescent characteristics without significant cell death. In the present study, the activity of SAβ-gal was measured by SAβ-gal staining at 24 h after H₂O₂ exposure. Approximately 75% of H₂O₂-exposed hMSCs were positive for SAβ-gal (blue cytoplasmic stain) (74.6±3.6%), whereas only 20% of the control cells without H₂O₂ exposure were SAβ-gal-positive (P<0.01) (Fig. 1A, B and E). However, the

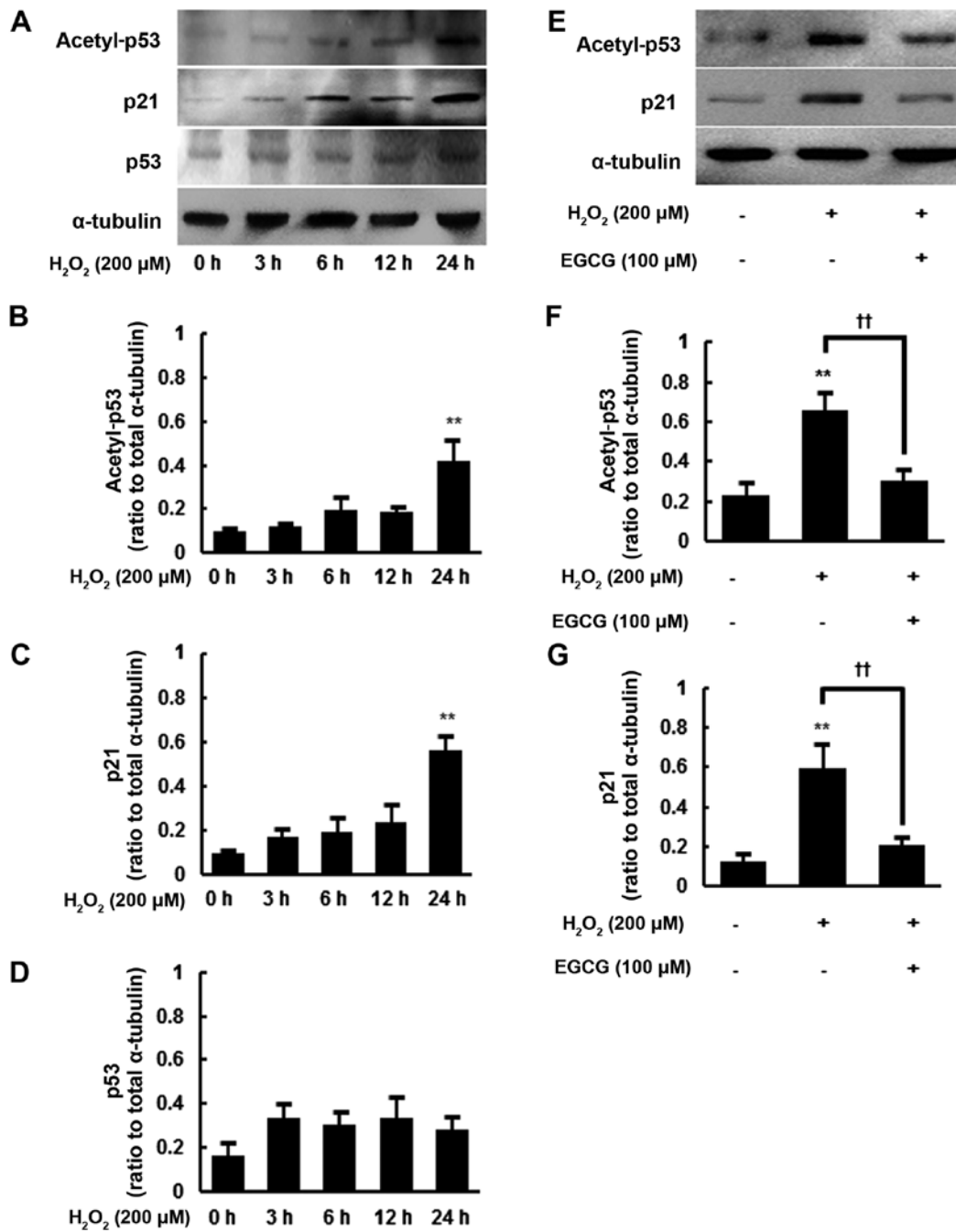


Figure 2. Epigallocatechin-3-gallate (EGCG) pre-treatment reduces H_2O_2 -induced increases in acetyl-p53 and p21 protein levels in human mesenchymal stem cells (hMSCs). (A-D) Western blot analysis and quantification of acetyl-p53, p21, and p53 protein levels in hMSCs at 0, 3, 6, 12 or 24 h after H_2O_2 (200 μ M) exposure for 2 h. (E-G) Western blot analysis and quantification of acetyl-p53 and p21 protein levels in hMSCs pre-treated with EGCG (100 μ M) for 6 h prior to H_2O_2 (200 μ M) exposure for 2 h. The values determined in four independent experiments are presented as the means \pm SEM. ** $P < 0.01$ vs. the control; †† $P < 0.01$ vs. H_2O_2 group. ANOVA followed by a post hoc Newman-Keuls test.

pre-treatment of hMSCs with 50 or 100 μ M EGCG for 6 h reduced the percentage of SA β -gal-positive cells following H_2O_2 exposure to 50.7 \pm 4.8 and 30.4 \pm 1.9%, respectively ($P < 0.01$) (Fig. 1C-E). Taken together, these results suggest that cellular senescence in hMSCs is accelerated by H_2O_2 exposure and EGCG pre-treatment reduces this acceleration in a dose-dependent manner. Furthermore, there were no significant differences in cell death among the experimental groups, indicating that H_2O_2 exposure induced cellular senescence without causing significant cell death (Fig. 1F).

EGCG pre-treatment reduces H_2O_2 -induced increases in acetylated p53 and p21 protein levels in hMSCs. To further evaluate H_2O_2 -induced changes in senescent cells, we next examined the protein levels of acetyl-p53, p53 and p21 in hMSCs at different times following 200 μ M H_2O_2 exposure. The expression of p53 and p21 is known to correlate with senescence in human primary cells and p53 acetylation has been shown to strongly promote cellular senescence (8,12). Consistent with the findings of previous studies, there were senescence-associated increases in the protein levels of acetyl-p53, p21 and p53 following

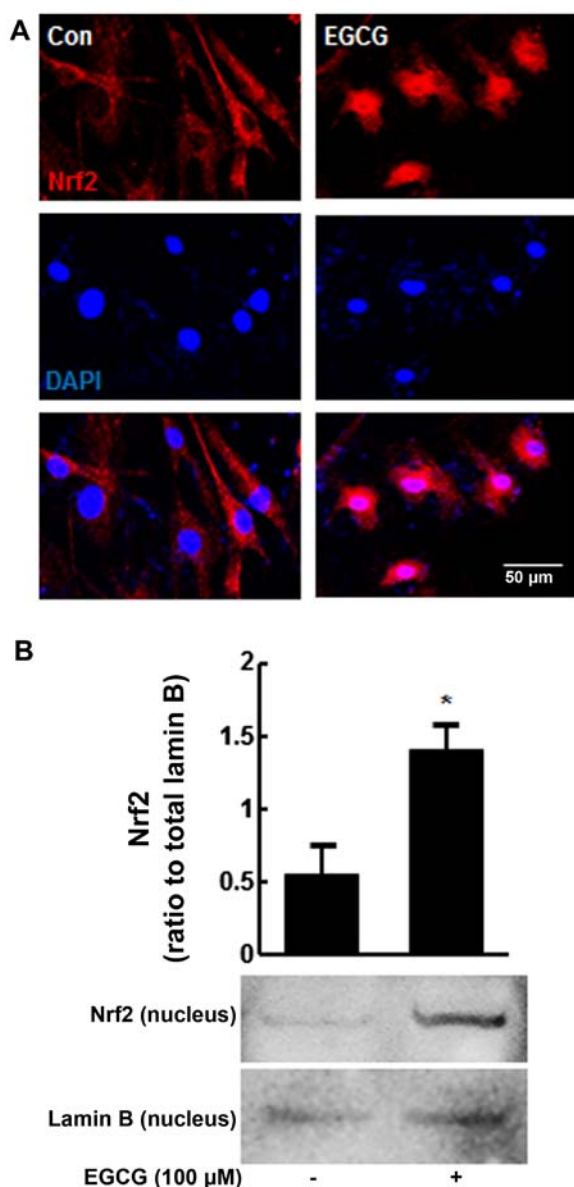


Figure 3. Epigallocatechin-3-gallate (EGCG) pre-treatment induces nuclear factor-erythroid 2-related factor 2 (Nrf2) translocation from the cytoplasm to the nucleus in human mesenchymal stem cells (hMSCs). (A) Immunocytochemical analysis of Nrf2 expression (red; top panels) and nuclear staining with DAPI (blue; middle panels) in hMSCs treated with or without 100 μM EGCG for 6 h (merge image, lower panels). Scale bar, 50 μm . (B) Western blot analysis and quantification of Nrf2 expression in the nuclear fraction. The nuclear envelope marker lamin B was used as a positive control of the nuclear fraction. Nrf2 protein levels determined in three independent experiments are presented as the means \pm SEM. * $P < 0.05$ vs. the control.

200 μM H_2O_2 exposure (Fig. 2A-D). Particularly after 24 h of H_2O_2 exposure, the protein levels of acetyl-p53 and p21 were significantly increased by up to 4.4- and 5.9-fold, respectively, compared with the controls, ($P < 0.01$) (Fig. 2B and C). Despite an increasing trend in total p53 protein levels following H_2O_2 exposure, the increase did not reach statistical significance (Fig. 2D).

Thus, we decided to examine the effect of EGCG pre-treatment on acetyl-p53 and p21 protein levels in hMSCs after 24 h of H_2O_2 exposure. As previously shown, there were

significant increases in acetyl-p53 and p21 protein levels at 24 h after 200 μM H_2O_2 exposure ($P < 0.01$) (Fig. 2E-G). However, EGCG pre-treatment (100 μM) significantly decreased the protein levels of acetyl-p53 and p21 in the H_2O_2 -exposed hMSCs by 46.3 \pm 8.1 and 35.1 \pm 6.5% ($P < 0.01$), respectively, compared with the cells given no EGCG pre-treatment (Fig. 2E-G).

EGCG induces nuclear translocation of Nrf2 in hMSCs. To determine whether the suppression of cellular senescence by EGCG in H_2O_2 -exposed hMSCs is associated with Nrf2 activation, we performed double-labeling experiments with anti-Nrf2 antibody and DAPI after 6 h of EGCG treatment (100 μM). Nrf2 was mostly found to be localized in the cytoplasm in the untreated cells (Fig. 3A, left panel). However, marked translocation of Nrf2 to the nuclei was observed after 6 h of EGCG treatment, although some Nrf2 remained in the cytoplasm (Fig. 3A, right panel). In addition, nuclear fractions were subjected to western blot analysis, showing that pre-treatment with EGCG increased nuclear Nrf2 protein levels 2.5-fold compared with the untreated cells ($P < 0.05$) (Fig. 3B).

EGCG pre-treatment suppresses H_2O_2 -induced cellular senescence and the expression of acetyl-p53 and p21 in hMSCs through Nrf2 activation. We hypothesized that Nrf2 activation may play an important role in the anti-senescence effects of EGCG. To test this hypothesis, we performed SA β -gal staining at 48 h after siRNA-mediated Nrf2 knockdown or control siRNA transfection (Fig. 4A). As previously shown in Fig. 1, the percentage of SA β -gal-positive cells in the 100 μM EGCG-pretreated/ H_2O_2 -exposed group was significantly reduced (35.1 \pm 2.5%) compared with the H_2O_2 -exposed cells without pre-treatment (78.4 \pm 3.7%) ($P < 0.01$) (Fig. 4A, panels b and c and 4B). However, EGCG-pre-treated and H_2O_2 -exposed/Nrf2-siRNA-transfected cells exhibited increased positive staining for SA β -gal (65.6 \pm 3.9%) ($P < 0.01$), which is similar to that of the H_2O_2 -exposed cells (Fig. 4A, panels b and d and 4B). By contrast, EGCG-pre-treated/ H_2O_2 -exposed/control-siRNA-transfected cells stained positive at a significantly lower rate of 36 \pm 4.2%, which is similar to that of the EGCG-pre-treated/ H_2O_2 -treated cells (Fig. 4A, panels c and e and 4B). We confirmed that Nrf2 protein levels were reduced to 30 \pm 5.4% at 48 h after Nrf2 siRNA transfection compared with the control siRNA ($P < 0.05$) (Fig. 4C). These results suggest that Nrf2 may play an important role in the anti-senescence activity of EGCG.

We next examined acetyl-p53 and p21 protein levels in Nrf2-knockdown hMSCs. As previously shown (Fig. 2E-G), acetyl-p53 and p21 protein levels were significantly reduced by 44.6 \pm 3.7 and 39.7 \pm 5.4%, respectively, in the EGCG-pre-treated/ H_2O_2 -exposed cells compared with the H_2O_2 -exposed cells ($P < 0.01$) (Fig. 4D-F). However, at 48 h after Nrf2-siRNA transfection, acetyl-p53 and p21 protein levels were significantly increased in the EGCG-pre-treated/ H_2O_2 -exposed cells. The protein levels of acetyl-p53 and p21 were similar to those in the H_2O_2 -exposed cells (Fig. 4D-F). By contrast, control siRNA transfection did not change the acetyl-p53 and p21 protein levels in the EGCG-pre-treated/ H_2O_2 -exposed cells. Taken together, these results indicate that Nrf2 activation by EGCG

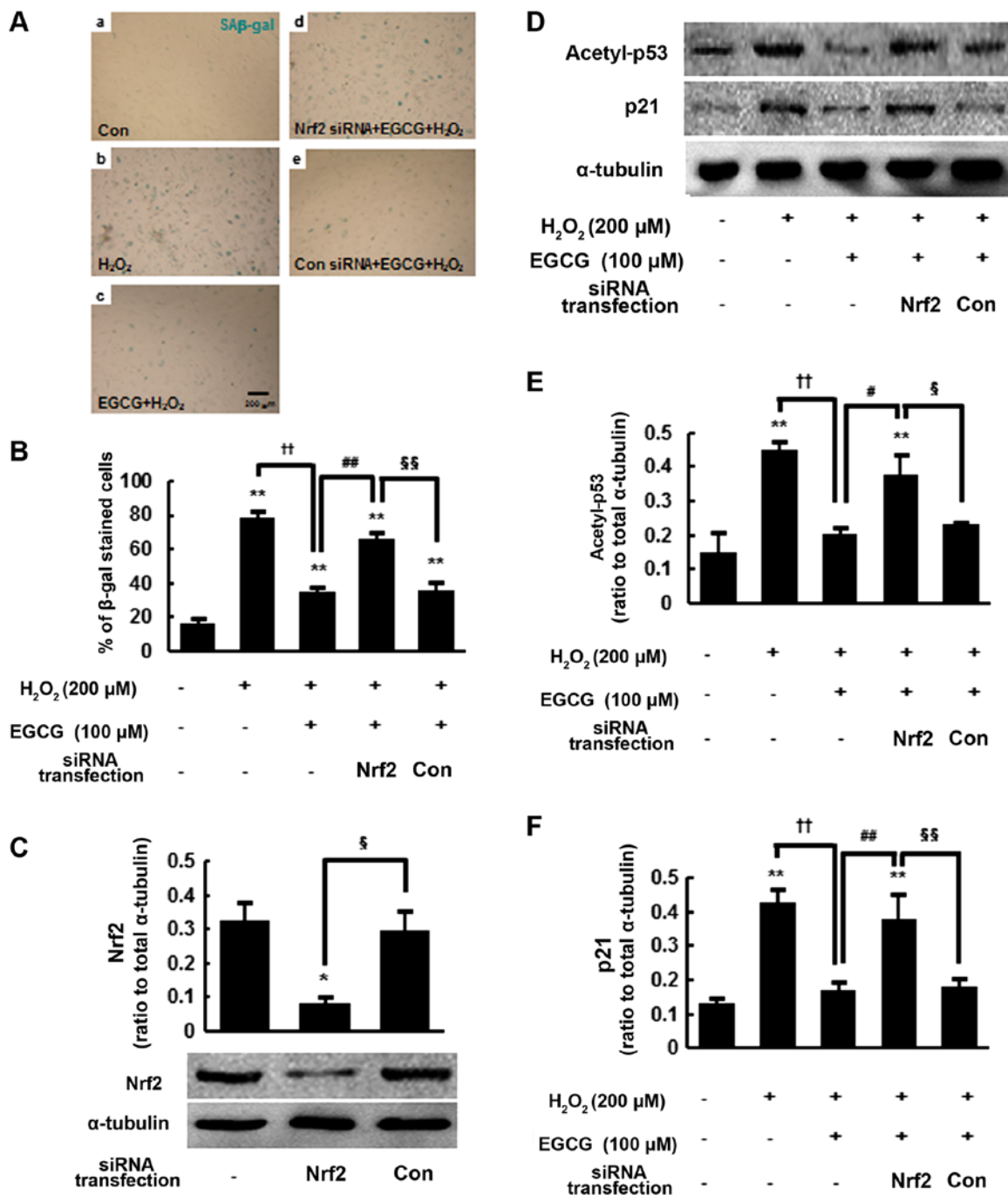


Figure 4. Nuclear factor-erythroid 2-related factor 2 (Nrf2) activation mediated by epigallocatechin-3-gallate (EGCG) pre-treatment suppresses H₂O₂-induced senescence and the expression of acetyl-p53 and p21 in human mesenchymal stem cells (hMSCs). (A) Senescence-associated β-galactosidase (SAβ-gal) staining analysis of control, H₂O₂-exposed, EGCG-pre-treated/H₂O₂-exposed, EGCG-pre-treated/H₂O₂-exposed/Nrf2-siRNA-transfected and EGCG-pre-treated/H₂O₂-treated/control-siRNA-transfected cells. hMSCs were transiently transfected for 48 h with either Nrf2 or control siRNA and treated with 100 μM EGCG for 6 h followed by H₂O₂ exposure (200 μM, 2 h). Twenty-four hours after H₂O₂ exposure, the cells were subjected to SAβ-gal staining (blue cytoplasmic stain). Scale bar, 200 μm. (B) Quantification of SAβ-gal activity. (C) Western blot analysis and quantification of Nrf2 at 48 h after Nrf2 siRNA or control siRNA transfection. (D-F) Western blot analysis and quantification of acetyl-p53 and p21 protein levels in each group. The levels determined in four independent experiments are presented as the means ± SEM. *P<0.05 and **P<0.01 vs. the control; ††P<0.01 vs. the H₂O₂ group. #P<0.05 and ##P<0.01 vs. the EGCG group; §P<0.05 and §§P<0.01 vs. the siNrf2 transfection group. ANOVA followed by a post hoc Newman-Keuls test.

pre-treatment suppresses H₂O₂-induced cellular senescence and the expression of acetyl-p53 and p21 in hMSCs (Fig. 5).

Discussion

The therapeutic applications of hMSCs are often limited by various factors, including senescence caused by the

inadequate culture conditions that affect their capacity for self-renewal and differentiation (3-5). Therefore, modulating hMSCs to block oxidative stress-induced cellular senescence may improve their clinical utility. Oxidative stress has been shown to induce cellular senescence as previously observed in human primary cells and hMSCs (6,12,21). In the present study, we also observed a significant increase in the number

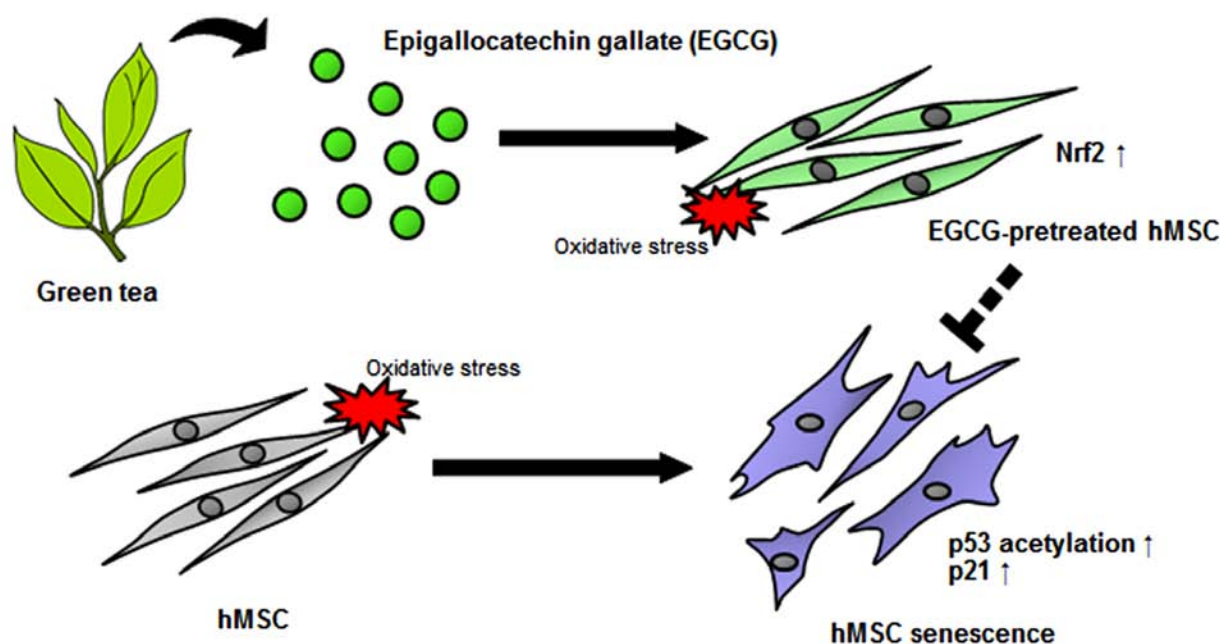


Figure 5. Diagram of novel molecular mechanisms underlying the anti-senescent effect of epigallocatechin-3-gallate (EGCG) in H_2O_2 -exposed human mesenchymal stem cells (hMSCs). EGCG prevents H_2O_2 -induced oxidative stress by upregulating nuclear factor-erythroid 2-related factor 2 (Nrf2) and downregulating the p53/p21 signaling pathway. Nrf2 activation by EGCG pre-treatment suppresses H_2O_2 -induced cellular senescence and the expression of acetyl-p53 and p21 in hMSCs, which suggests a potential role for Nrf2 in p53/p21 regulation to thereby prevent oxidative stress-induced cellular senescence in hMSCs.

of SA β -gal-positive hMSCs following H_2O_2 exposure, which induces cellular senescence by generating intracellular ROS (3,4).

EGCG, a polyphenol, is a strong neutralizing agent of excessive ROS and induces Nrf2 expression (17). Nrf2 plays an important role in the cellular antioxidant defense system by activating the expression of antioxidant and detoxifying genes, such as superoxide dismutase, heme oxygenase 1, and glutathione S-transferases. These genes have been shown to protect cells against oxidative stress caused by ROS by restoring redox homeostasis and inhibiting oxidative damage (20). A recent study has reported that EGCG suppressed H_2O_2 -mediated oxidative stress in hMSCs (16). Consistently, our results also demonstrated that EGCG prevented H_2O_2 -induced senescence in hMSCs.

ARE-mediated antioxidant gene expression is a widely accepted model for the activity of EGCG (20). In general, the serine/threonine residues of Nrf2 are phosphorylated by protein kinases such as PI3K, ERK, p38 and JNK thereby enhancing the nuclear translocation of Nrf2 and subsequent ARE binding. Oxidized or other reactive forms of EGCG conjugate with glutathione (GSH) and decrease cellular GSH concentrations, which leads to a disruption of the redox state and the activation of upstream protein kinases, triggering Nrf2 phosphorylation. It is also plausible that EGCG may oxidize or modify specific cysteine thiol groups in Keap1 that allow the nuclear translocation of Nrf2. We observed the marked translocation of Nrf2 into the nuclei after EGCG treatment (Fig. 3). Both of these are plausible mechanisms for EGCG-induced Nrf2 activation as electrophilic agents or compounds have been reported to interact with cysteine residues directly and stimulate Nrf2 dissociation (22).

p53 acetylation has been shown to promote cellular senescence in addition to activating growth suppressive genes (23,24). The first confirmed downstream target of p53, p21, is an essential regulator of p53-dependent cell cycle arrest which leads to cell cycle arrest in response to DNA damage. As a cyclin-dependent kinase inhibitor, p21 regulates the function of cyclin D1/CDK4 and cyclin E/CDK2 complexes and induces the accumulation of hypophosphorylated Rb, which leads to Rb binding with E2F transcription factors, resulting in cell cycle arrest (25,26). In addition, previous studies have shown that p21 is a key regulator of cellular senescence in human primary cells (27,28).

Recent studies have challenged the known paradigm of Nrf2. The inhibition of Nrf2 by caveolin-1, a structural protein of caveolae, reduces its cellular antioxidant response following H_2O_2 exposure (29). The inhibition of Nrf2 also suppresses the expression of murine double minute (Mdm2), an oncogene which promotes p53 degradation, resulting in p53 pathway activation (30). In addition to the Keap1-Nrf2 complex formation, caveolin-1 and/or Mdm2 may be candidates responsible for modulating p53 acetylation and p21 activation in hMSCs in response to oxidative stress. However, further studies are warranted in order to elucidate the physiological relevance of these mechanisms.

In conclusion, our results are consistent with the hypothesis that Nrf2 activation inhibits oxidative stress in cells. The upregulation of Nrf2 by EGCG prevented oxidative stress-induced cellular senescence through the downregulation of p53 acetylation and p21 in hMSCs. These findings demonstrate that EGCG is capable of increasing Nrf2 activation in hMSCs and suggest a novel approach for preventing the oxidative stress-induced cellular senescence of human stem cells.

Acknowledgements

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References

- Pittenger MF1, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S and Marshak DR: Multilineage potential of adult human mesenchymal stem cells. *Science* 284: 143-147, 1999.
- Sherman LS, Munoz J, Patel SA, Dave MA, Paige I and Rameshwar P: Moving from the laboratory bench to patients' bedside: considerations for effective therapy with stem cells. *Clin Transl Sci* 4: 380-386, 2011.
- Kuilman T, Michaloglou C, Mooi WJ and Peeper DS: The essence of senescence. *Genes Dev* 24: 2463-2479, 2010.
- López-Otín C, Blasco MA, Partridge L, Serrano M and Kroemer G: The hallmarks of aging. *Cell* 153: 1194-1217, 2013.
- Oh J, Lee YD and Wagers AJ: Stem cell aging: mechanisms, regulators and therapeutic opportunities. *Nat Med* 20: 870-880, 2014.
- Brandl A, Meyer M, Bechmann V, Nerlich M and Angele P: Oxidative stress induces senescence in human mesenchymal stem cells. *Exp Cell Res* 317: 1541-1547, 2011.
- Campisi J and d'Adda di Fagagna F: Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol* 8: 729-740, 2007.
- Kume S, Haneda M, Kanasaki K, Sugimoto T, Araki S, Isono M, Isshiki K, Uzu T, Kashiwagi A and Koya D: Silent information regulator 2 (SIRT1) attenuates oxidative stress-induced mesangial cell apoptosis via p53 deacetylation. *Free Radic Biol Med* 40: 2175-2182, 2006.
- Langley E, Pearson M, Faretta M, Bauer UM, Frye RA, Minucci S, Pelicci PG and Kouzarides T: Human SIR2 deacetylates p53 and antagonizes PML/p53-induced cellular senescence. *EMBO J* 21: 2383-2396, 2002.
- Luo J, Nikolaev AY, Imai S, Chen D, Su F, Shiloh A, Guarente L and Gu W: Negative control of p53 by Sir2 α promotes cell survival under stress. *Cell* 107: 137-148, 2001.
- Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, Guarente L and Weinberg RA: hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* 107: 149-159, 2001.
- Han DW, Lee MH, Kim B, Lee JJ, Hyon SH and Park JC: Preventive effects of epigallocatechin-3-O-gallate against replicative senescence associated with p53 acetylation in human dermal fibroblasts. *Oxid Med Cell Longev* 2012: 850684, 2012.
- Anderson RF, Fisher LJ, Hara Y, Harris T, Mak WB, Melton LD and Packer JE: Green tea catechins partially protect DNA from (.)OH radical-induced strand breaks and base damage through fast chemical repair of DNA radicals. *Carcinogenesis* 22: 1189-1193, 2001.
- Chow HH, Cai Y, Alberts DS, Hakim I, Dorr R, Shahi F, Crowell JA, Yang CS and Hara Y: Phase I pharmacokinetic study of tea polyphenols following single-dose administration of epigallocatechin gallate and polyphenon E. *Cancer Epidemiol Biomarkers Prev* 10: 53-58, 2001.
- Higdon JV and Frei B: Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Crit Rev Food Sci Nutr* 43: 89-143, 2003.
- Yagi H, Tan J and Tuan RS: Polyphenols suppress hydrogen peroxide-induced oxidative stress in human bone-marrow derived mesenchymal stem cells. *J Cell Biochem* 114: 1163-1173, 2013.
- Surh YJ, Kundu JK, Na HK and Lee JS: Redox-sensitive transcription factors as prime targets for chemoprevention with anti-inflammatory and antioxidative phytochemicals. *J Nutr* 135 (Suppl 12): 2993S-3001S, 2005.
- Hayes JD and McMahon M: NRF2 and KEAP1 mutations: permanent activation of an adaptive response in cancer. *Trends Biochem Sci* 34: 176-188, 2009.
- Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD and Yamamoto M: Keap1 represses nuclear activation of anti-oxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev* 13: 76-86, 1999.
- Nguyen T, Nioi P and Pickett CB: The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J Biol Chem* 284: 13291-13295, 2009.
- Burova E, Borodkina A, Shatrova A and Nikolsky N: Sublethal oxidative stress induces the premature senescence of human mesenchymal stem cells derived from endometrium. *Oxid Med Cell Longev* 2013: 474931, 2013.
- Dinkova-Kostova AT, Holtzclaw WD and Wakabayashi N: Keap1, the sensor for electrophiles and oxidants that regulates the phase 2 response, is a zinc metalloprotein. *Biochemistry* 44: 6889-6899, 2005.
- Bond J, Haughton M, Blydes J, Gire V, Wynford-Thomas D and Wyllie F: Evidence that transcriptional activation by p53 plays a direct role in the induction of cellular senescence. *Oncogene* 13: 2097-2104, 1996.
- Luo J, Li M, Tang Y, Laszkowska M, Roeder RG and Gu W: Acetylation of p53 augments its site-specific DNA binding both in vitro and in vivo. *Proc Natl Acad Sci USA* 101: 2259-2264, 2004.
- el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B: WAF1, a potential mediator of p53 tumor suppression. *Cell* 75: 817-825, 1993.
- Harper JW, Adami GR, Wei N, Keyomarsi K and Elledge SJ: The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 75: 805-816, 1993.
- Brown JP, Wei W and Sedivy JM: Bypass of senescence after disruption of p21CIP1/WAF1 gene in normal diploid human fibroblasts. *Science* 277: 831-834, 1997.
- Herbig U, Wei W, Dutriaux A, Jobling WA and Sedivy JM: Real-time imaging of transcriptional activation in live cells reveals rapid up-regulation of the cyclin-dependent kinase inhibitor gene CDKN1A in replicative cellular senescence. *Aging Cell* 2: 295-304, 2003.
- Volonte D, Liu Z, Musille PM, Stoppani E, Wakabayashi N, Di YP, Lisanti MP, Kensler TW and Galbiati F: Inhibition of nuclear factor-erythroid 2-related factor (Nrf2) by caveolin-1 promotes stress-induced premature senescence. *Mol Biol Cell* 24: 1852-1862, 2013.
- You A, Nam CW, Wakabayashi N, Yamamoto M, Kensler TW and Kwak MK: Transcription factor Nrf2 maintains the basal expression of Mdm2: an implication of the regulation of p53 signaling by Nrf2. *Arch Biochem Biophys* 507: 356-364, 2011.