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Pelargonidin reduces the TPA induced transformation of mouse epidermal cells –potential involvement of Nrf2 promoter demethylation

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Abstract

Pelargonidin, a well-known natural anthocyanidin found in berries strawberries, blueberries, red radishes and other natural foods, has been found to possess health beneficial effects including anti-cancer effect. Herein, we investigated the effect of pelargonidin on cellular transformation in mouse skin epidermal JB6 (JB6 P+) cells induced by tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). Pelargonidin treatment significantly decreased colony formation and suppressed cell viability of JB6 P+ cells. Pelargonidin also induced the anti-oxidant response element (ARE)-luciferase activation in HepG2-C8 cells overexpressing the ARE-luciferase reporter. Knockdown of nuclear factor E2-related factor 2 (Nrf2) in shNrf2 JB6 P+ cells enhanced TPA-induced colony formation and attenuated pelargonidin's blocking effect. Pelargonidin reduced the protein levels of genes encoding methyltransferases (DNMTs) and histone deacetylases (HDACs). Importantly, pelargonidin decreased the DNA methylation in the Nrf2 promoter region of JB6 P+ cells and increased Nrf2 downstream target genes expression, such as NAD(P)H/quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1), involved in cellular protection. In summary, our results showed that pelargonidin blocks TPA-induced cell transformation. The possible molecular mechanisms of its potential anti-cancer effects against neoplastic transformation may be attributed to its activation of Nrf2-ARE signaling pathway and its cytoprotective effect.

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Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Keywords

pelargonidin; epigenetics; Nrf2; antioxidant; mouse epidermal cells

1. Introduction

Anthocyanidins are well-known and powerful antioxidants that have been applied in the treatment of various disorders induced by oxidative stress [1]. Pelargonidin (pelargonidin chloride chemical structure is shown in Fig. 1) is one type of anthocyanidin, which are plant pigments that are found in vegetables and fruits, such as red radishes [2] and berries, including lingonberries, cranberries, saskatoon berries, chokeberries, blueberries and strawberries [3–5]. Pelargonidin has also been detected in pomegranate [6] and kidney beans [7]. Pelargonidin exerts various biological activities including antioxidant [8], anti-inflammatory [9], antithrombotic [10], and anti-diabetic [11]. Furthermore, the chemopreventive potential of pelargonidin has been investigated in a cell model, in which it upregulated the activities and levels of detoxification enzymes to block reactive oxygen species (ROS) [8]. However, the underlying antioxidant mechanism of pelargonidin remains poorly understood.

Nuclear factor E2-related factor 2 (Nrf2) is an important transcription factor that protects against damage induced by oxidative stress [12]. Nrf2 is transported into the nucleus in response to oxidative stress to activate the expression of many antioxidative stress genes by binding to the antioxidant response element (ARE) region [13]. In unstressed conditions, the Nrf2 level is very low, and is mainly located in the perinuclear cytoplasm through a negative regulator of Kelch-like ECH-associated protein 1 (KEAP1) in normal cells. However, activated Nrf2 translocates to the nucleus, where it binds to ARE and induces transcription of many cytoprotective genes under oxidative stress caused by ROS and toxic chemicals [14, 15]. Importantly, aberrant accumulation of Nrf2 has been reported in Nrf2-addicted cancer cells through disrupted binding of KEAP1 to Nrf2 [15, 16]. Aberrant Nrf2 activation promotes cell proliferation and cancer progression, and contributes to therapy resistance [16]. Previous studies have also reported that Nrf2 plays an important role in resistance to oxidative stress and chemical-induced damage, as verified by Nrf2-deficient mice [17, 18]. Recent research has indicated that many dietary natural compounds, such as triterpenoids, isothiocyanates, and polyphenols, exert anti-inflammatory, anti-tumor and antioxidation effects by activating the Nrf2-ARE pathway [19].

Epigenetic regulation is emerging as an important mechanism for controlling phenotypic gene expression and is potentially involved in many diseases, including cancer [20–24]. Evidence suggests that epigenetic mechanisms may lead to chromatin remodeling and genomic instability via histone status and DNA methylation [25]. In recent years, many natural compounds possessing cancer chemopreventive effects were also shown to elicit epigenetic effects [21]. Dietary phytochemicals have been shown to modify DNA methyltransferases (DNMTs) and histone deacetylases (HDACs), which could contribute to the regulation of epigenetic modification [26]. Hypermethylation of the KEAP1 promoter have been reported to be associated with KEAP1 downregulation and aberrant Nrf2

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activation in lung cancer [27]. In our previous studies, dietary phytochemicals activates the Nrf2-ARE pathway, induces demethylation of Nrf2 promoter and decreases protein levels of DNMTs and HDACs [22, 28–30]. Thus, it is important to understand how bioactive dietary components can induce DNA methylation changes and chromatin alterations associated with gene expression [21, 31].

So far, however, there has been little discussion about pelargonidin in the Nrf2 activation associated with skin cells. Mouse skin epidermal JB6 (JB6 P+) cells are sensitive to transformation by tumor-promoting agents such as 12-O-tetradecanoylphorbol-13-acetate (TPA) [28]. By topical application of TPA in vivo onto the skin, TPA can induce oxidative stress, increase ear thickness, weight and inflammatory cytokines [32–34]. Moreover, TPA promotes the expression of oncogene REGγ through the MAPK/p38/AP-1 signaling pathway and protein kinase C (PKC) and activates Wnt/β-catenin pathway, which is important for the initiation and progression of skin carcinogenesis [35]. In our current study, we evaluated whether the pelargonidin can decrease neoplastic transformation caused by TPA in mouse epidermal JB6 P+ cells. We also explored the underlying mechanisms by which pelargonidin exerts its effects against cell transformation, including the Nrf2-ARE pathway and epigenetic modifications.

2. Materials and Methods

2.1. Reagents

Pelargonidin chloride was purchased from Alkemist labs (Costa Mesa, CA, USA). 5-aza-deoxycytidine (5-aza), Eagle's basal medium, trichostatin A (TSA) and TPA were provided by Sigma-Aldrich (St. Louis, MO, USA). The CellTiter 96® AQueous One Solution Reagent (MTS) and luciferase activity assay kit were provided by Promega (Madison, WI, USA). Primary antibodies specific for actin, Nrf2, NQO1, and HO1 were provided by Santa Cruz Biotechnology (CA, USA). Specific antibodies for HDAC1, HDAC2, HDAC3, HDAC4 and HDAC6, DNMT1, DNMT3a and DNMT3b were provided by Cell Signaling Technology (Beverly, MA, USA). The TOPO TA Cloning Kit was provided by Invitrogen (Invitrogen, USA).

2.2. Cell Culture and Treatment

JB6 P+ cells, HepG2-C8 cells, and JB6 P+ cells transfected with the shMock (wild type) and shNrf2 (knockdown) virus-mediated short hairpin RNAs (shRNAs) were maintained as described in our previous reports [22, 28]. After overnight incubation, the medium was changed to include different concentrations of pelargonidin in MEM supplemented with 1% FBS. Treatment with 250 nmol/L 5-aza (an inhibitor of DNA methyltransferases) and 50 nmol/L TSA (an inhibitor of histone deacetylases) served as positive control. TSA was only added on day 4. DMSO (0.1%) was used as a vehicle control. The treatment agents were changed every other day.

2.3. Cell Viability and Proliferation Assay

First, JB6 P+ cells (3×10^3 cells per well) were maintained in 96-well plates for 24 hours. Then, the cells were exposed to either DMSO (0.1%) or pelargonidin (10, 30, 50, 70 and 100

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μM) in MEM supplemented with 1% FBS. The cell culture medium containing pelargonidin was changed every other day. To determine cell viability and proliferation, 20 μL of MTS reagent was added to each well of the 96-well plates after 1, 3, or 5 days of treatment. The absorbance at 490 nm was recorded after 1 hour of incubation.

2.4. Cell Cycle Analysis

Briefly, the JB6 P+ cells were incubated in 100-mm dishes with 5% FBS/MEM at a density of 3×10^5 cells per dish and allowed to adhere via overnight incubation. After treatment with various concentrations of pelargonidin (10, 30, 50, 70 and 100 μM) in 1% FBS for 3 days, the cells were collected and fixed with chilled 66% ethanol. DMSO (0.1%) was used as a vehicle control. The cells were stained by propidium iodide flow cytometry kit (cat no. ab139418, Abcam, Cambridge, MA, USA). The stained cells were further analyzed by Beckman Coulter Gallios Flow Cytometer. The cell cycle distributions were recorded by Gallios software during sample acquisition. Data were analyzed from three parallel experiments.

2.5. Anchorage-independent Cell Transformation Assay

This assay was based on the established TPA-induced neoplastic transformation assay, as described previously [22, 30]. Cells (4×10^4 cells per well) were pretreated with TPA (20 ng/mL) or TPA plus pelargonidin in 6-well plates for 3 days. Then, the pretreated cell suspension (8,000 cells/well) and 0.66% agar were mixed at a 1:1 ratio. The pretreated cells were incubated with TPA or TPA with different concentrations of pelargonidin in soft agar for an additional 2 weeks. Imaging was performed by a Nikon ACT-1 system, and colonies in soft agar were counted by ImageJ analysis software.

2.6. Luciferase Reporter Activity Assay

HepG2-C8 Cells were established from immortalized HepG2 cell line by transfection with a plasmid containing pARE-TI-luciferase to investigate the potency of pelargonidin in activating the Nrf2 pathway. The HepG2 cells are adherent, epithelial-like cells derived from a liver hepatocellular carcinoma, which are used as a tool for the exploration of cytoprotective, antigenotoxic and cogenotoxic agents [36]. HepG2-C8 cells (1×10^5 cells per well) were cultured in 12-well plates overnight. Then, the cells were treated with DMSO (0.1%) or different concentrations of pelargonidin for 24 hours. Sulforaphane (SFN) served as a positive control. After the cells in each well were lysed by the reporter lysis buffer, 10μL of cell lysate with 50μL of luciferase assay reagent was analyzed, according to the protocol described in our previous report [28].

2.7. Western Blotting

JB6 P+ cells (3×10^5 cells per 100-mm dish) were cultured overnight. Then, the cells were incubated with pelargonidin (10, 30 and 50 μM) and 0.1% DMSO (control) in MEM supplemented with 1% FBS. Total protein from the control group and treatment groups was extracted using sonication in RIPA lysis buffer supplemented with a protease inhibitor cocktail, as described previously [22]. Subsequently, the proteins were transferred and detected by antibodies specific for NQO1 (1:1000), HO1 (1:1000), Nrf2 (1:1000), DNMT1

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(1:2000), DNMT3a (1:2000), DNMT3b (1:2000), HDAC1 (1:2000), HDAC2 (1:2000), HDAC3 (1:2000), HDAC4 (1:2000), HDAC6 (1:2000) and actin (1:3000). The signal band intensity was calculated using ImageJ software. The relative expression level was calculated based on the intensity of each band normalized to β-actin.

2.8. Quantitative Real-time Polymerase Chain Reaction

Cells (3×10^5 cells per 100-mm dish) were incubated with pelargonidin (10, 30 and 50 μM), and 0.1% DMSO (control) in MEM containing 1% FBS. RNA was extracted from each group by GeneJET RNA Purification Kits (Thermo Fisher Scientific, Rockford, IL). Then, 1 μg of RNA was used for reverse transcription by Taqman Reverse Transcription Reagents (Thermo Fisher Scientific, Rockford, IL). Gene expression was measured on the transcription level by quantitative real-time PCR on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Rockford, IL) and quantified by the ΔΔCt method. The data from triplicate experiments were subjected to statistical analysis. The primers are listed as follows: Nrf2: 5'-GGCTCAGCACCTTGTATCTT-3' and 5'-CACATTGCCATCTCTGGTTTG-3'; NQO1: 5'-GAGAAGAGCCCTGATTGTACTG-3' and 5'-ACCTCCCATCCTCTCTTCTT-3'; GAPDH: 5'-AACAGCAACTCCCACTCTTC-3' and 5' -CCTGTTGCTGTAGCCGTATT-3'; and HO-1: 5'-CTCCCTGTGTTTCCTTTCTCTC-3' and 5'-GCTGCTGGTTTCAAAGTTCAG-3'.

2.9. Bisulfite Genomic Sequencing

This assay was performed based on our previous report [22]. Briefly, JB6 P+ cells (3×10^5 cells per 100-mm dish) were incubated overnight. Then cells were incubated with pelargonidin (10, 30 and 50 μM), 0.1% DMSO (control) or 5-aza (500 nM) in 1% FBS MEM for 5 days. The 5-aza cotreatment with TSA served as positive control group. TSA (100 nM) was only added on day 4. The combination of 5-aza with TSA could generate greater effects on the cell growth and DNA synthesis than either agent alone [37]. Several reports have shown that TSA also can directly or indirectly affect DNA methylation [38, 39]. Genomic DNA was collected on day 5. Then, the DNA (500 ng) was subjected to bisulfite conversion using the EZ DNA Methylation Gold Kit. The murine Nrf2 promoter sequence from −863 to −1226 was amplified using specific primers. The specific sequences were as follows: sense, 5′-AGTTATGAAGTAGTAGTAAAAA-3′; antisense, 5′-ACCCCAAAAAAATAAATAAATC-3′. Then, the extracted PCR products were cloned into pCR4 TOPO vectors. At least 10 colonies per group from three independent experiments were randomly picked and the sequences were analyzed using T7 primers by Genewiz (South Plainfield, NJ, USA).

2.10. Statistical Analysis

All values are expressed as the mean ± SD of at least three independent replicates. Statistical analysis between groups was performed by Student's *t*-test. *, $P< 0.05$ and **, $P< 0.01$ were considered to be statistically significant.

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3. Results

3.1. Cytotoxicity of Pelargonidin

First, to measure the cytotoxicity of pelargonidin, mouse epidermal JB6 P+ cells were treated with pelargonidin (0, 10, 30, 50, 70 and 100 μM) dissolved in DMSO (vehicle). Cell viability assays were performed on days 1, 3 and 5. Treatment with pelargonidin resulted in time- and dose-dependent effects on cell viability (Fig. 2A). The cell viabilities following pelargonidin (< 50 μM) treatment were above 70% after the 1-day, 3-day and 5-day treatments. Hence, those doses were selected for subsequent experiments.

3.2. Effects of Pelargonidin on the Cell Cycle

To further investigate the effects of pelargonidin on growth, the cell cycle was measured using flow cytometry. As shown in Fig. 2B, the cell cycle distributions of JB6 P+ cells were not obviously altered by treatment with pelargonidin on day 3. However, the presence of apoptotic nuclei was found, as indicated by increases in the hypodiploid cell population. The sub-G1 phase cells showed a dose-dependent response to pelargonidin treatment (Fig. 2B). The proportion of cells in the sub-G1 phases on day 3 in the control group was 3.75 ± 0.45%, whereas those in the pelargonidin groups (10, 30 and 50 μM) were 6.90 ± 2.96%, 7.98 ± 1.58%, 8.42 ± 0.68%, respectively.

3.3. Pelargonidin Inhibits TPA-induced JB6 P+ Cells Transformation

To determine whether pelargonidin possess chemopreventive and anticancer effects, we examined the effect of pelargonidin on TPA-induced cell transformation in soft agar for two weeks. The TPA treatment group exhibited a 5.8-fold change in transformed cell colonies compared with the control group, indicating that TPA can induce epidermal JB6 P+ cell transformation (Fig. 3). Compared with the TPA treatment group, pelargonidin treatment at various concentrations ranging from 0 to 50 μM, significantly reduced the number of transformed cell colonies (Fig. 3). These data suggest that pelargonidin has the potential to against TPA-induced cell transformation in epidermal JB6 P+ cells.

3.4. Pelargonidin Induces ARE-Luciferase Reporter Activity

We investigated the efficacy of pelargonidin in inducing ARE-luciferase reporter expression in HepG2-C8 cells that were established by transfection with pARE-TI-luciferase to investigate the potency of pelargonidin in activating the Nrf2-ARE pathway. The results showed that pelargonidin induced luciferase expression in a dose-dependent manner, with upregulations of approximately 1.7-fold and 3-fold after pelargonidin treatment (50 and 100 μM, respectively) compared to the control group (Fig. 4). The luciferase activity also increased after treatment with the SFN (10 μM) positive control, as expected. This finding suggests that pelargonidin can reactivate the Nrf2 pathway and subsequently regulate the transcription of antioxidant/detoxification genes.

3.5. Pelargonidin Increases the Gene Expression of Nrf2 Target Genes

Next, to further evaluate the ability of pelargonidin to reactivate Nrf2 and its downstream genes, qPCR and western blotting were performed in JB6 P+ cells. Our results show that the

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mRNA expression levels of HO-1 and NQO1 increased in a dose-dependent manner when the cells were treated with pelargonidin for 6 hours (Fig. 5A) and5 days (Fig. 5B). In addition, pelargonidin significantly elevated the protein levels of the Nrf2 target genes HO-1 and NQO1 after 5 days of treatment. Together, these findings suggest that pelargonidin has the ability to increase the Nrf2 downstream genes expression in JB6 P+ cells.

3.6. The Knockdown of Nrf2 Reduces Resistance to TPA-induced Colony Formation

To test the impact of Nrf2 on TPA-induced anchorage-independent growth, shMock and shNrf2 JB6 P+ cell lines were used, as described in our previous reports [22, 28]. The results showed that treatment with pelargonidin suppressed colony formation in both shMock and shNrf2 JB6 P+ cells (Fig. 6). Compared to shMock cells, knockdown Nrf2 in shNrf2 JB6 P+ cells enhanced TPA-induced colony formation and attenuated pelargonidin's blocking effect. As shown in Fig. 6, pelargonidin at concentrations of 0, 10, 30, and 50 μM reduced the colony formation frequency of shMock cells to 6.3%, 5.3%, 4.4% and 1.6%, respectively. Compared to shMock cells, pelargonidin at a concentration of 50 μM mediated an inhibitory effect on the colony formation frequency of shNrf2 cells, with an increase from 1.6% to 4.2% (Fig. 6). These findings demonstrate that Nrf2 plays an important role in the preventive effect of pelargonidin on TPA-induced colony formation.

3.7. Pelargonidin Decreases DNA Methylation of the Nrf2 Promoter

To examine whether the Nrf2 promoter was epigenetically modified by pelargonidin in JB6 P+ cells, we explored the DNA methylation status of the mouse Nrf2 promoter located from −1226 to−863 by bisulfite genomic DNA sequencing, as previously reported [22, 30]. Bisulfite genomic sequencing showed that the proportion of methylated CpG sites significantly decreased after treatment with pelargonidin. As shown in Fig. 7, 88.5 ± 6.6% of sites in the promoter of Nrf2 were methylated in the control group. However, after treatment with 10 μM, 30 μM and 50 μM pelargonidin for 5 days, the proportion of methylated sites was reduced to 85.6 ± 2.0%, 83.6 ± 2.7%, and 76.5 ± 4.1%, respectively. The cells treated with the combination of 5-aza (an inhibitor of DNA methyltransferases) and TSA (an inhibitor of histone deacetylases), had a decreased DNA methylation rate of 70.0 ± 5.7% (Fig. 7), which is similar to that described in our previous report [29]. This finding suggests that pelargonidin can decrease the DNA methylation rate in the Nrf2 promoter region.

3.8. Pelargonidin Decreases Protein Levels of DNMTs and HDACs

To explore the potential molecular epigenetic mechanisms by which pelargonidin reduced DNA methylation in the Nrf2 promoter, we further measured the effects of pelargonidin on protein levels of DNMTs and HDACs. Treatment with pelargonidin caused a significant decrease in DNMT1 and DNMT3b expression in JB6 P+ cells (Fig. 8A). DNMT3a protein levels were not significantly affected by pelargonidin treatment. Since, gene expression can be affected or modified by histone de-acetylation and acetylation, Wang et al [40] reported that histone deacetylase inhibitor TSA could induce acetylation of Nrf2 and activates transcription factor Nrf2, although may or may not affect CpG methylation per se. Here, we observed that the levels of HDACs were also decreased by pelargonidin treatment (Fig. 8B). Among the HDACs, the levels of HDAC1, HDAC2, HDAC3, HDAC4, and HDAC7 were significantly reduced by pelargonidin treatment. This finding suggests that pelargonidin can

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decrease protein levels of DNMTs and HDACs, which may potentially contribute to the DNA demethylation of the Nrf2 promoter region.

4. Discussion

Numerous studies have reported that bioactive dietary components with therapeutic potential exert effects in disease prevention by immunomodulation [41], epigenetic modifications [26, 42], cellular metabolism [43], inhibition of inflammation and cell cycle alterations [44]. Recently, anthocyanidins, which are pigments in fruits and vegetables, have received increased attention because they exhibit tumor cell proliferation inhibitory activity [45, 46]. Pelargonidin, cyanidin, malvidin, delphinidin, peonidin, and petunidin all belong to the bioflavonoid anthocyanidins [47]. Pelargonidin, the most abundant anthocyanidin found in strawberries and radish, has multiple protective effects against many diseases by preventing or decreasing inflammation and oxidative stress [8, 9]. Here, we showed that pelargonidin can reduce cell transformation caused by TPA in epidermal cells in vitro. Furthermore, the possible molecular mechanisms for the ability of pelargonidin against transformation can be attributed to its activity in the Nrf2-ARE signaling pathway through demethylation of Nrf2 promoter and its cytoprotective effect.

The ROS produced by oxidative stress could destroy cellular components and subsequently impact cell apoptosis and proliferation, invasiveness and metastasis [48, 49]. Fruits and vegetables that are rich in antioxidants can help reduce the harm induced by excess ROS [50]. Nrf2 and its interaction with AREs increase the transcription of phase II detoxifying/antioxidant enzymes, such as superoxide dismutase (SOD), HO-1, and NQO1, which are indispensable for preventing oxidative stress and cancer initiation [51, 52]. We demonstrate that treatment with pelargonidin increases ARE-luciferase expression in HepG2-C8 cells stably transfected with the ARE-luciferase reporter gene. In addition, pelargonidin promoted the mRNA and protein levels of HO-1 and NQO1 after 6 hours and 5 days of treatment. TPA has often been employed as an inducer for endogenous superoxide production and as a potent skin carcinogenesis promoter and is routinely used to activate the signal transduction enzyme protein kinase C in biomedical studies [53–55]. We observed that colony formation induced by TPA was suppressed by pelargonidin treatment. Furthermore, our previous studies indicated that expressing the antioxidant genes HO-1 and NQO1 by reactivation of the Nrf2 pathway appeared to be important for the reduction of TPA-induced cell transformation [22, 28, 56]. To further demonstrate the function of Nrf2 in cell transformation, we generated Nrf2 knockdown JB6 P+ cell lines by virus-mediated short hairpin RNAs. The inhibition of colony growth in soft agar by pelargonidin treatment was attenuated through the ablation of Nrf2 expression in JB6 P+ shNrf2 cells. In the absence of Nrf2, pelogonidin, may not able to activate some of the Nrf2 pathway genes and therefore, may not be able to block of some the TPA-induced ROS/inflammatory driven cellular transformation machinery.

Genomic hypermethylation of DNA within CpG islands is observed in cancers [57, 58]. Many dietary components have exhibited important effects in regulating these epigenetic modifications [59]. Bioactive natural compounds with chemoprevention activities mediated by epigenetic regulation are regarded as potential therapeutic drugs against human disease.

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This evidence is also consistent with our previous observations, which showed that bioactive natural compounds reduced the methylation of the CpGs of Nrf2 compared with that of the control group [22, 28, 30]. In this study, our study also showed that pelargonidin reduced the DNA methylation of the Nrf2 promoter region in the first 15 CpG sites (Fig. 7). Compared with the control, 50 μM pelargonidin decreased DNA methylation, similar to the positive control 5-aza+TSA, which significantly reduced the methylation of CpG sites, as expected, suggesting that activation of the Nrf2-ARE signaling pathway is associated with the demethylation of CpG sites. Enzymes in the DNA methyltransferase family, including DNMT1, DNMT3A, and DNMT3B, catalyze the transfer of a methyl group to DNA at the 5′ position of the cytosine residue within CpG dinucleotides [60]. We observed that pelargonidin can decrease the expression of DNMT1 and DNMT3b (Fig. 8). Furthermore, we also observed that the protein levels of the histone deacetylases HDAC1, HDAC2, HDAC3, HDAC4 and HDAC7 were significantly reduced by pelargonidin treatment in a dose-dependent manner (Fig. 8). HDAC inhibitors have been reported as potential anticancer drugs previously [61]. Our results revealed that the antioxidant effects of pelargonidin are closely correlated with its ability to decrease protein levels of DNMTs and HDACs, which is the key link to DNA demethylation in the Nrf2 promoter region.

In conclusion, our results show that pelargonidin can inhibit neoplastic transformation caused by TPA in skin epidermal cells. Moreover, the possible molecular mechanisms of the chemopreventive effects of pelargonidin against neoplastic transformation can be attributed to activation of the Nrf2-ARE defense pathway through demethylation of Nrf2 promoter and its cytoprotective effect.

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Abbreviations

TPA	12-O-tetradecanoylphorbol-13-acetate
ARE	anti-oxidant response element
Nrf2	nuclear factor E2-related factor 2
DNMTs	DNA methyltransferases
HDACs	histone deacetylases
HO-1	heme oxygenase-1
NQO1	NAD(P)H/quinone oxidoreductase 1
KEAP1	Kelch like-ECH-associated protein 1
SOD	superoxide dismutase
ROS	reactive oxygen species

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Highlights

- Anthocyanin pelargonidin attenuates colony formation of JB6 mouse epidermal cells
- Pelargonidin transcriptionally activates the antioxidant response element (ARE)
- Pelargonidin enhances Nrf2-mediated antioxidant gene expression
- Knockdown of Nrf2 attenuates blocking effect of pelargonidin on colony formation
- Pelargonidin epigenetically decreases the DNA CpG methylation of Nrf2 promoter

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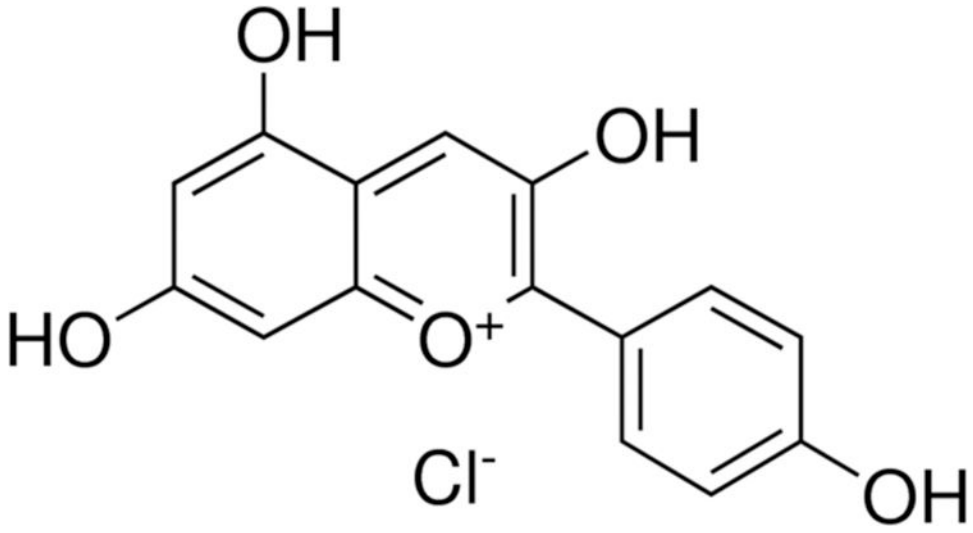

Fig. 1.
Chemical structure of pelargonidin chloride.

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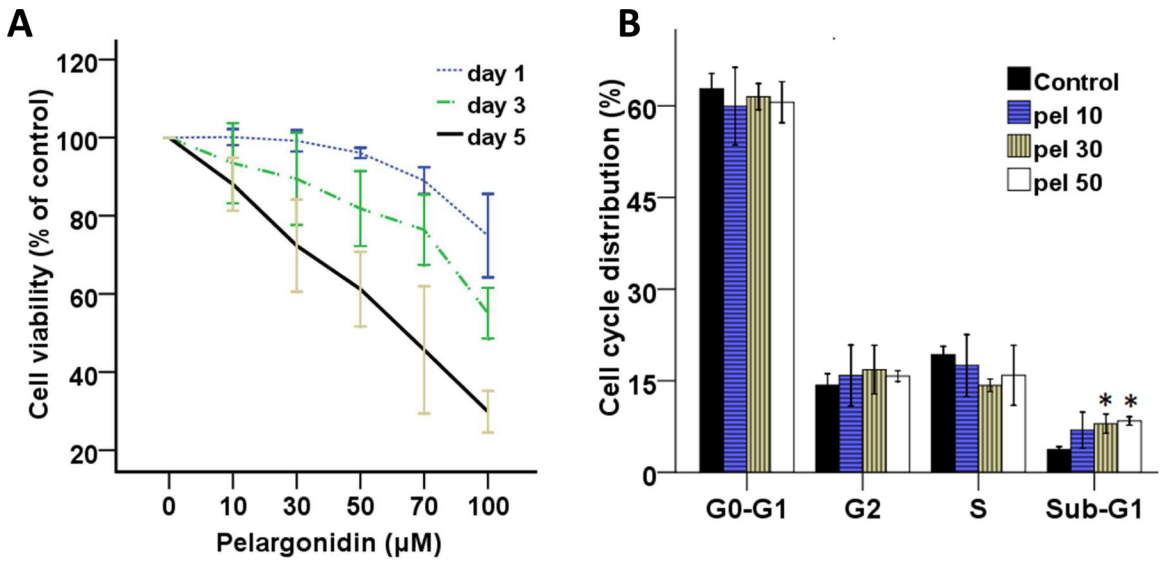

Fig. 2.

Effects of pelargonidin on cell growth in vitro. (A) Cell viability of JB6 P+ cells after treatment with pelargonidin. (B) Percentage of JB6 P+ cells in different phases of the cell cycle after treatment with pelargonidin (10, 30 and 50 μM) for 3 days. *, $P < 0.05$ compared to the control group.

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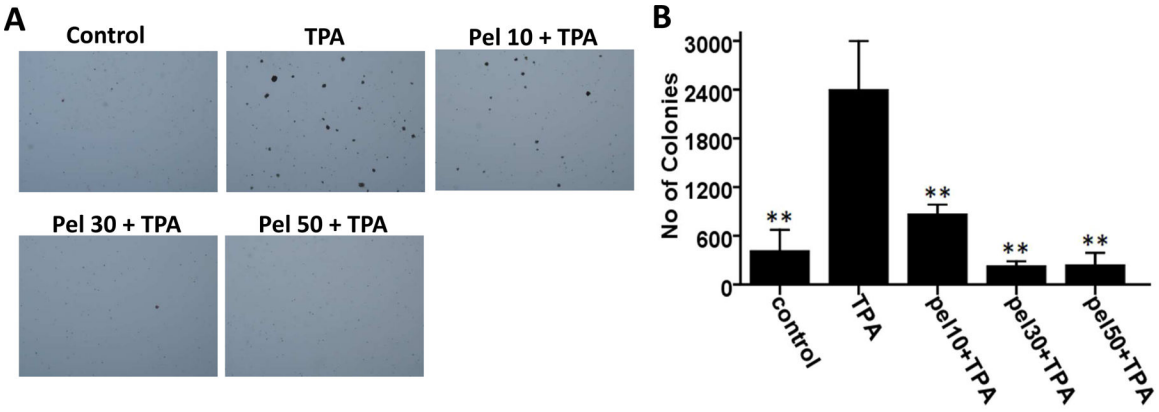

Fig. 3.

Pelargonidin inhibits TPA-induced cell transformation. (A) Representative images of colony growth in soft agar. (B) The statistical results are presented as the mean ± SD of three independent replicates. **, $P < 0.01$ indicates significant differences between the treatment and TPA alone groups. Student's t-test was performed to calculate significant differences.

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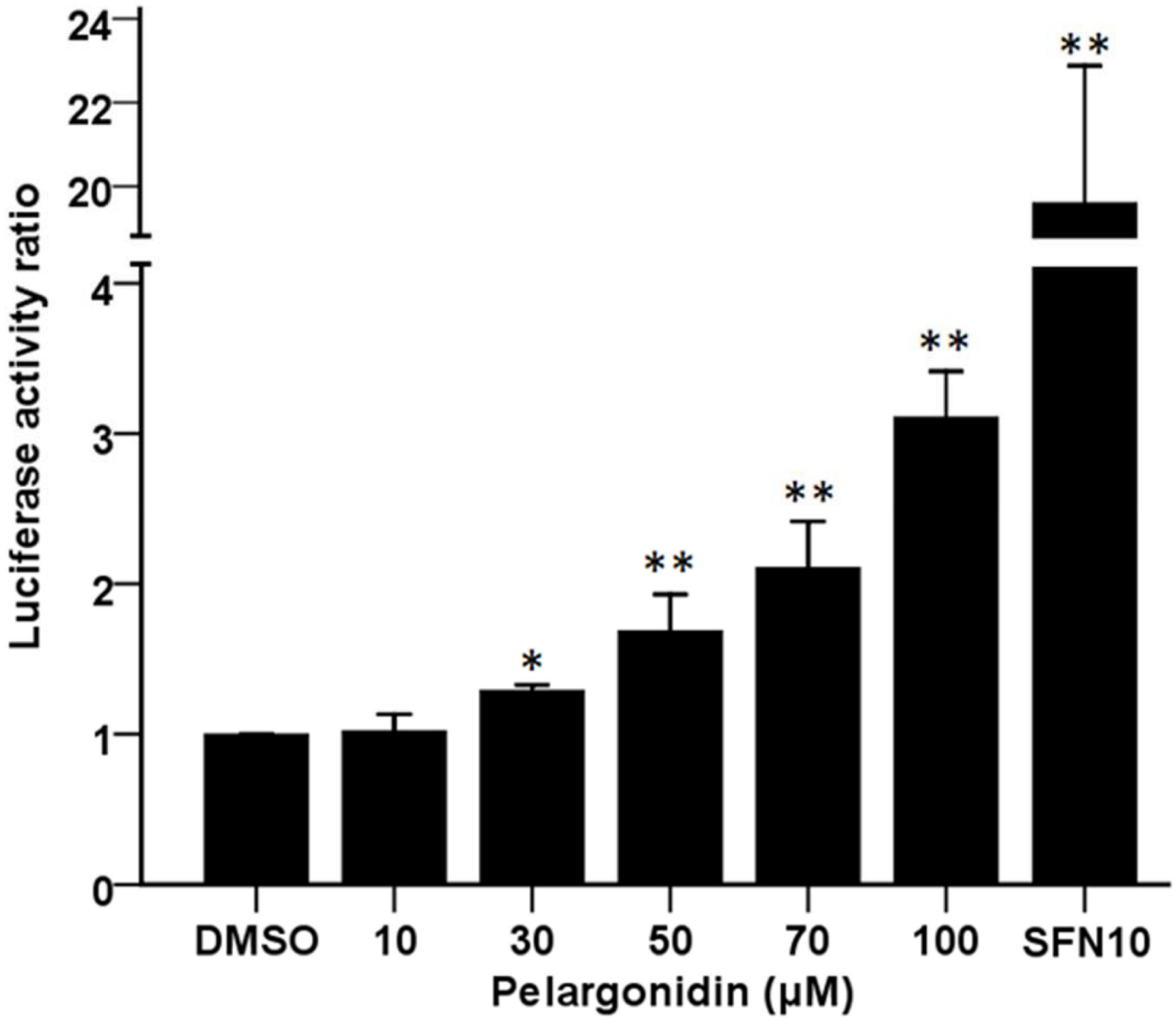

Fig. 4.

Increase in ARE-luciferase expression in response to pelargonidin in HepG2-C8 cells. Luciferase expression was calculated based on the protein concentrations in each group. The statistical data are presented as the mean ± SD of three independent treatments in HepG2-C8 cells. *, $P< 0.05$ and **, $P< 0.01$, compared to the control group.

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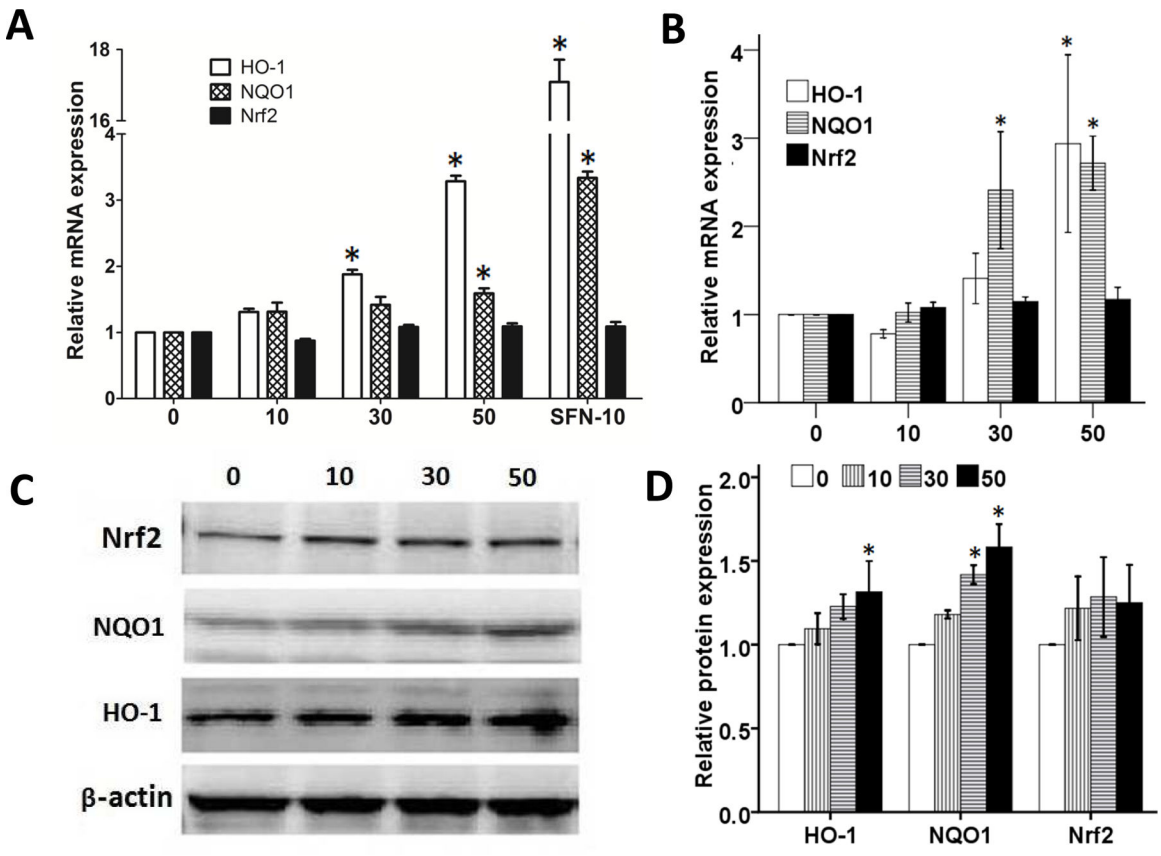

Fig. 5.

Effect of pelargonidin on the expression of Nrf2 and its target genes in JB6 P+ cells. Pelargonidin significantly promoted the mRNA expression of HO-1 and NQO1 after 6 hours (A) and 5 days of treatment (B). (C) Protein levels of Nrf2, NQO1, β-actin and HO-1 after 5 days of treatment with various concentrations of pelargonidin were determined by immunoblot analyses. (D) The relative expression level was calculated based on the intensity of each band normalized to β-actin. The statistical data are presented as the mean ± SD of three independent experiments. *, $P < 0.05$ compared to the control group.

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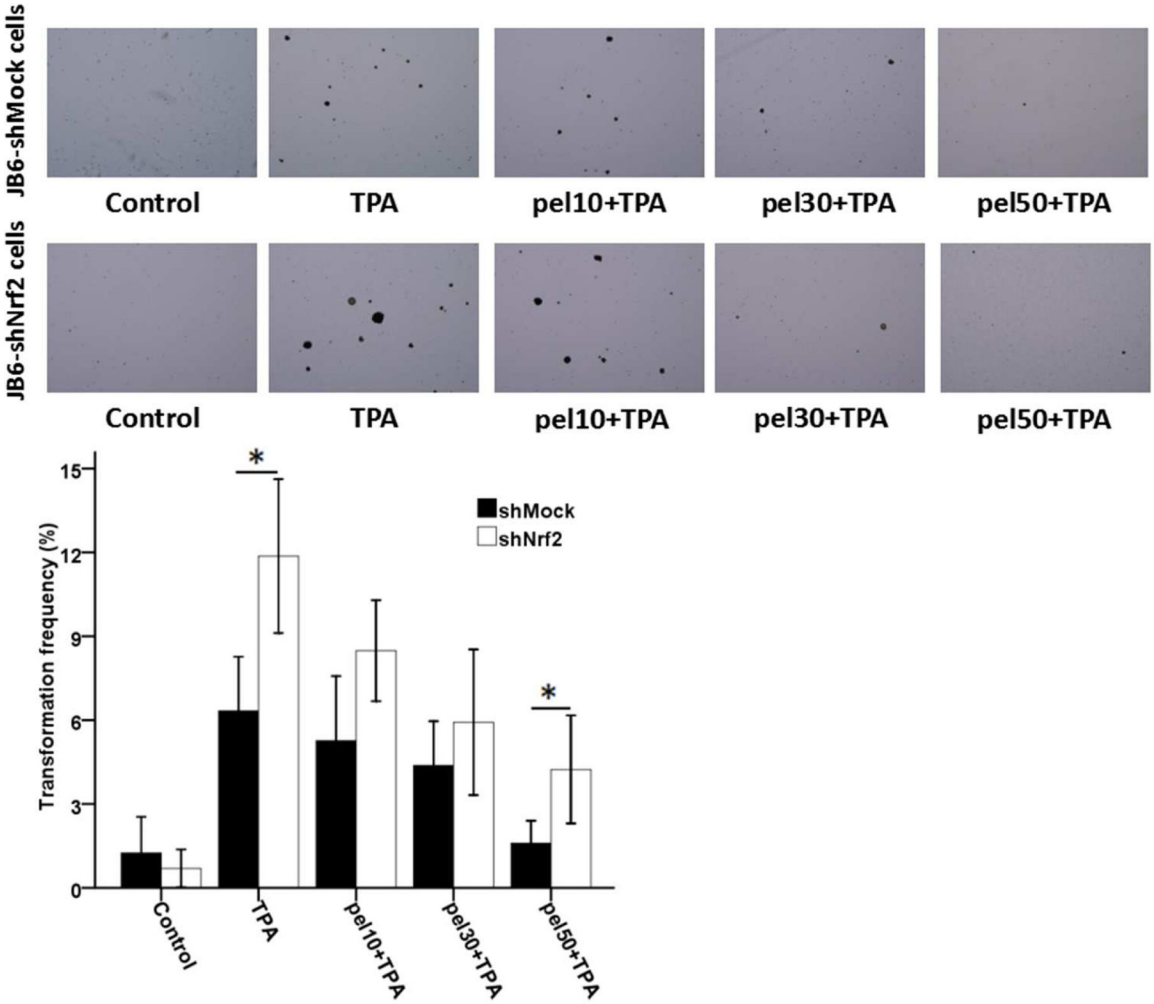

Fig. 6.

Inhibitory effects of pelargonidin on the cell transformation of stably transfected shNrf2 and shMock JB6 P+ cells. (A) Representative images of colony growth in soft agar. (B) The incidence of cell transformation was decreased during pelargonidin treatment. The transformation frequency data are presented as the mean ± SD of three independent replicates. *, $P < 0.01$ indicates significant differences between the shNrf2 and shMock groups. Student's t-test was performed to calculate significant differences.

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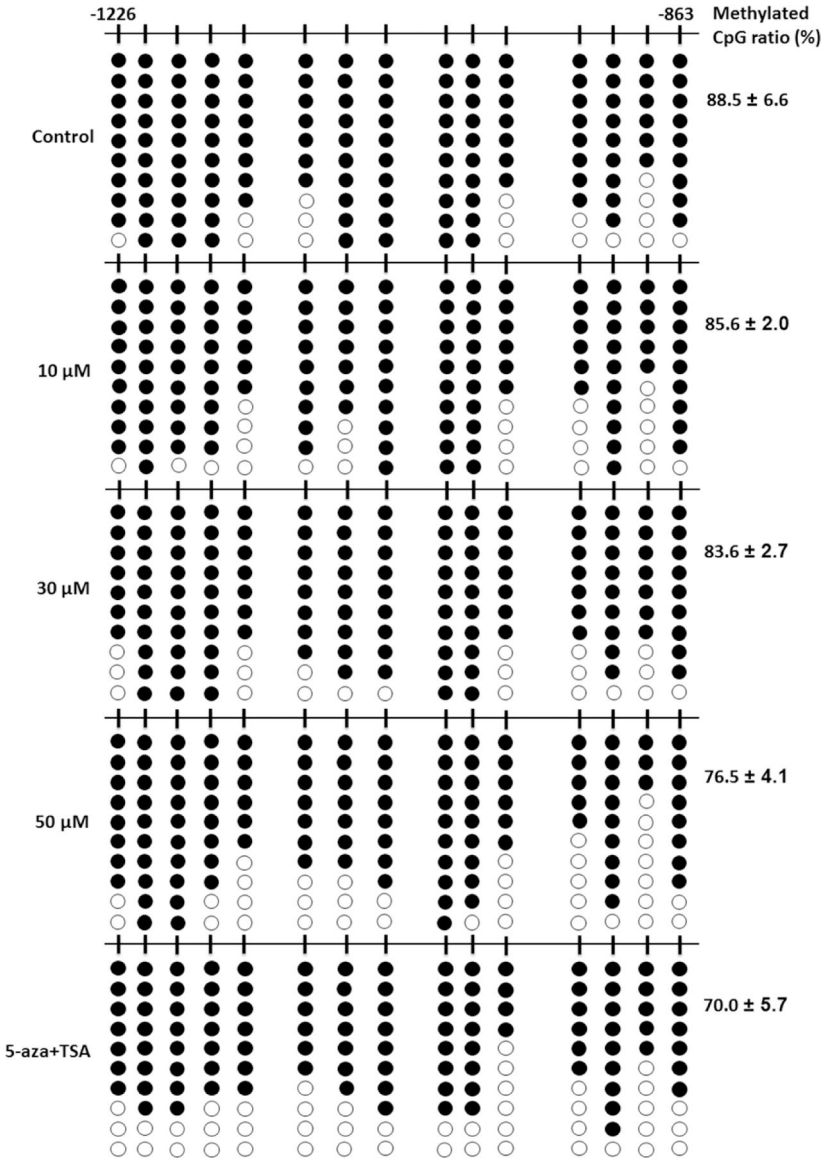

Fig. 7.

Pelargonidin decreases DNA methylation of the Nrf2 promoter region. DNA was extracted from pelargonidin- and 5-aza/TSA-treated JB6 P+ cells after 5 days. The methylation level at the 15 CpG sites (located between −1226 and −863 relative to the transcriptional start site) in the Nrf2 promoter region was determined by bisulfite genomic sequencing. Black spots indicate methylated CpGs, and hollow dots indicate unmethylated CpGs. At least 10 clones from each group were sequenced using T7 primers. The data are shown as the percentage of methylated cytosines among a total of 15 CpGs from three individual experiments.

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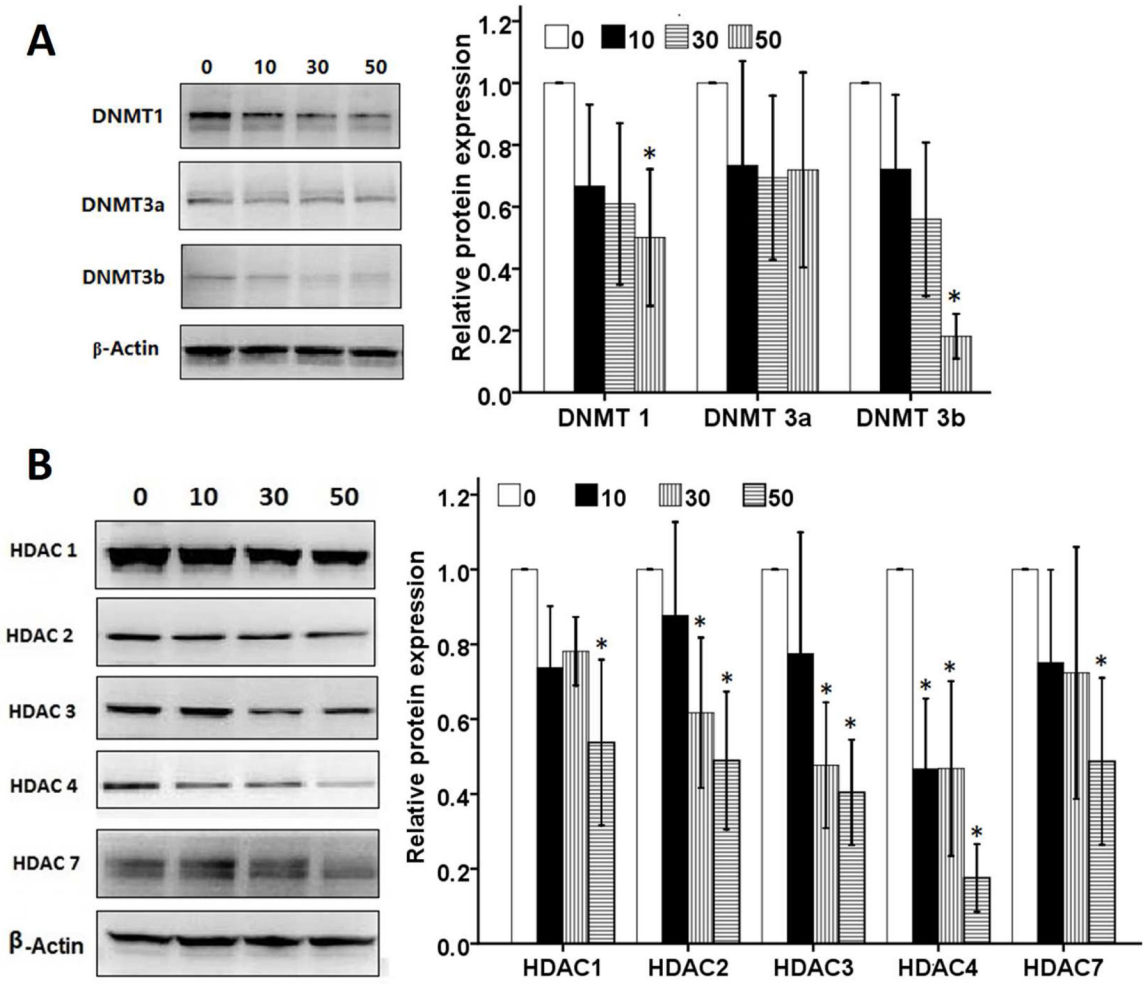

Fig. 8.

Effect of pelargonidin on protein levels of DNMTs and HDACs in JB6 P+ cells. (A) The protein levels of DNMT1, DNMT3a and DNMT3b after 5 days of treatment with various concentrations of pelargonidin was determined by immunoblot analyses. (B) The protein levels of HDAC1, HDAC2, HDAC3, HDAC4 and HDAC7 after 5 days of treatment with various concentrations of pelargonidin was determined by immunoblot analyses. The statistical data are presented as the mean ± SD of three independent experiments. *, $P < 0.05$ compared to the control group.