Published OnlineFirst January 17, 2014; DOI: 10.1158/1940-6207.CAPR-13-0313-T

Research Article

Cancer Prevention Research

Requirement and Epigenetics Reprogramming of Nrf2 in Suppression of Tumor Promoter TPA-Induced Mouse Skin Cell Transformation by Sulforaphane

Zheng-Yuan Su[1,2], Chengyue Zhang[1,2], Jong Hun Lee[1,2], Limin Shu[1,2], Tien-Yuan Wu[1,2], Tin Oo Khor[1,2], Allan H. Conney[1,3,4,†], Yao-Ping Lu[1,3], and Ah-Ng Tony Kong[1,2]

Abstract

Nrf2 is a transcription factor that plays critical roles in regulating the expression of cellular defensive antioxidants and detoxification enzymes. However, the role of Nrf2 and Nrf2's epigenetics reprogramming in skin tumor transformation is unknown. In this study, we investigated the inhibitory role and epigenetics of Nrf2 on tumor transformation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in mouse skin epidermal JB6 (JB6 P+) cells and the anticancer effect of sulforaphane (SFN), an isothiocyanate found in cruciferous vegetables. After five days of treatment, SFN significantly inhibited TPA-induced JB6 cellular transformation and SFN enhanced the nuclear translocation of Nrf2 and increased the mRNA and protein levels of the Nrf2 target genes HO-1, NQO1 and UGT1A1. Knockdown of Nrf2 attenuated the induction of Nrf2, HO-1 and NQO1 by SFN, enhanced TPA-induced colony formation and dampened the inhibitory effect of SFN on TPA-induced JB6 transformation. Epigenetics investigation using bisulfite genomic sequencing showed that SFN decreased the methylation ratio of the first 15 CpGs of the Nrf2 gene promoter, which was corroborated by increased Nrf2 mRNA expression. Furthermore, SFN strongly reduced the protein expression of DNA methyltransferases (DNMT1, DNMT3a and DNMT3b). SFN also inhibited the total histone deacetylase (HDAC) activity and decreased the protein expression of HDAC1, HDAC2, HDAC3 and HDAC4. Collectively, these results suggest that the anti-cancer effect of SFN against TPA-induced neoplastic transformation of mouse skin could involve the epigenetic reprogramming of anti-cancer genes such as Nrf2, leading to the epigenetic reactivation of Nrf2 and the subsequent induction of downstream target genes involved in cellular protection. *Cancer Prev Res; 7(3); 319–29. ©2014 AACR.*

Introduction

An increase in oxidative stress may result in genomic instability, genetic mutation, and neoplastic transformation, increasing the incidence of carcinogenesis (1). Endogenous reactive oxygen species (ROS) can be generated during normal cellular metabolism, immune reactions, and several pathologic conditions, including inflammation, whereas exogenous ROS may originate from air pollution, UV irradiation, microorganisms, viruses, and xenobiotics. Therefore, skin is a major organ that is subjected to exogenous ROS and endogenous oxidative stress, which may trigger skin carcinogenesis (2). Nuclear factor erythroid-2-related factor-2 (Nrf2 or NFE2L2) is a basic helix-loop-helix leucine zipper transcription factor that plays a key role in reducing cellular oxidative stress by regulating the defense system (3, 4). Nrf2 itself is regulated by Kelch-like ECH-associating protein 1 (Keap1), which serves as an adaptor protein that bridges Nrf2 and Cul3, resulting in the ubiquitination of Nrf2 (5). By binding to an antioxidant response element (ARE) in the promoter region of a target gene, nuclear Nrf2 induces the expression of antioxidative stress/detoxifying enzymes, such as heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase-1 (NQO-1), UDP-glucuronosyltransferase (UGT), and glutathione-*S*-transferase (GST; 6, 7). It has been reported that the susceptibility to tumorigenesis induced by a carcinogen is higher in Nrf2-deficient mice (8, 9), and cancer chemoprevention is partially correlated with the induction of phase II enzymes (10, 11). Our recent study demonstrated that the expression of Nrf2 can be regulated by epigenetic alterations in both the prostate tissue of the transgenic adenocarcinoma of the mouse prostate (TRAMP) model and tumorigenic TRAMP C1 cells (12).

In addition to genetic changes, accumulating evidence suggests that carcinogenesis is regulated by epigenetic

Authors' Affiliations: [1]Center for Cancer Prevention Research; Departments of [2]Pharmaceutics and [3]Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, New Jersey; and [4]Allan H. Conney Laboratory for Anticancer Research, Guangdong University of Technology, Guangzhou, China

†Deceased.

Corresponding Author: Ah-Ng Tony Kong, Center for Cancer Prevention Research, Department of Pharmaceutics, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, 160 Frelinghuysen Road, Piscataway, New Jersey 08854. Phone: 732-455-3831; Fax: 732-455-3134; E-mail: KongT@pharmacy.rutgers.edu

doi: 10.1158/1940-6207.CAPR-13-0313-T

Published OnlineFirst January 17, 2014; DOI: 10.1158/1940-6207.CAPR-13-0313-T

alterations in tumor suppressor genes and oncogenes, defined as gene expression that can be regulated without the alteration of DNA sequences (13, 14). DNA methylation and histone modification, managed by DNA methyltransferases (DNMT) and histone deacetylases (HDAC), respectively, can maintain cellular DNA stability and integrity and are 2 major epigenetic mechanisms for the transcriptional control of gene promoters. Drugs targeting the enzymes responsible for epigenetic silencing are continually being researched and developed as cancer chemopreventive/therapeutic agents, including 5-azadeoxycytidine (5-aza, a DNMT inhibitor) and trichostatin A (TSA, an HDAC inhibitor; refs. 15 and 16). However, owing to the side effects of such drugs, many dietary phytochemicals have recently been investigated for their cancer preventive potential and have been shown to induce epigenetic alterations (17, 18). For example, epigallocatechin-3-gallate (EGCG) from green tea has been reported to be an inhibitor of DNMTs and histone acetyltransferases (HAT; refs. 19 and 20). Our previous studies also demonstrate that curcumin from *Curcuma longa* and Z-ligustilide from Radix *Angelica sinensis* might induce the expression of Nrf2 in the TRAMP C1 cell line via an inhibitory effect on the activity of DNMTs (21, 22).

Sulforaphane (SFN) is a natural isothiocyanate found in cruciferous vegetables, including broccoli, cabbage, cauliflower, Chinese cabbage, and watercress. The cancer chemopreventive potential of SFN has been investigated in numerous animal models of different cancers, and one of the major mechanisms of SFN is to induce several phase II antioxidant and detoxification enzymes to block electrophilic chemicals that cause DNA damage, mutagenesis, and carcinogenesis (23). It has been reported that the topical application of an SFN-containing broccoli sprout extract can inhibit UVB-induced skin carcinogenesis in SKH-1 mice (24). SFN can also suppress skin tumorigenesis caused by 7,12-dimethylbenz(a)anthracene (DMBA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA) in C57BL/6 mice, an effect that is potentially mediated through the regulation of Nrf2 (25). SFN also protects mouse skin from mustard gas analog 2-(chloroethyl) ethyl sulfide–induced mutagenesis through the induction of phase II detoxification enzymes (26). Moreover, these results suggest that epigenetic modification, including the inhibition of HDAC activity, may contribute to the multitargeted chemopreventive activity of SFN (27). Our recent study also found that SFN reactivates Nrf2 in TRAMP C1 cells, a result that involves a decrease in DNMT and HDAC expression and gene promoter demethylation (28). However, the molecular mechanisms by which SFN inhibits the skin carcinogenesis promoted by tumor promoters such as TPA, which may be mediated by epigenetic alteration, have not yet been clearly described. In this study, we investigated whether SFN can inhibit neoplastic transformation induced by TPA, which promotes skin tumor development, in cultured mouse skin epidermal JB6 P+ cells. Furthermore, the possible molecular mechanisms, including Nrf2-mediated pathways and epigenetic modification, were also investigated to aid in the understanding of the chemopreventive effect of SFN against skin carcinogenesis.

Materials and Methods

Materials

SFN was purchased from LKT Laboratories, Inc. 5-Aza, bacteriological agar, Eagle's basal medium (BME), puromycin, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and TSA were obtained from Sigma-Aldrich. FBS, minimum essential medium (MEM), and trypsin-EDTA solution were purchased from Gibco Laboratories.

Cell culture and treatment

JB6 P+ (JB6 Cl 41-5a, ATCC CRL-2010) cells from American Type Culture Collection were maintained in MEM containing 5% FBS at 37°C in a humidified 5% CO_2 atmosphere. JB6 P+ cells stably transfected with shMock- and shNrf2-knockdown were maintained in MEM supplemented with 5% FBS and 2 μg/mL puromycin. After incubation for 24 hours, the cells were treated with various concentrations of SFN or 5-aza (250 nmol/L) in MEM containing 1% FBS for 5 days. The medium was changed every 2 days. For the combination treatment of 5-aza and TSA, TSA (50 nmol/L) was added to the medium on day 4. The treated cells were harvested on day 5 for further assays.

Protein fraction preparation

Protein supernatant fractions from whole-cell lysates were prepared from the treated cells using radioimmunoprecipitation assay buffer (Cell Signaling) supplemented with a protease inhibitor cocktail (Sigma). For preparation of the nuclear protein fraction, the NEPER Nuclear and Cytoplasmic Protein Extraction Kit (Thermo Scientific) was used according to the manufacturer's instructions. The protein concentrations were determined using the Bicinchoninic Acid (BCA) Kit (Pierce).

Western blotting

Proteins separated by 4% to 15% SDS-PAGE (Bio-Rad) were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). After blocking with 5% BSA in Tris-buffered saline-0.1% Tween 20 buffer, the membrane was sequentially incubated with specific primary antibodies and horseradish peroxidase–conjugated secondary antibodies. The SuperSignal enhanced chemiluminescence detection system and Gel Documentation 2000 system (Bio-Rad) were used to detect and record the antibody-bound proteins on the membrane, and the densitometry of the bands was analyzed using the ImageJ program (Version 1.40g; NIH). The primary anti-Nrf2, anti-HO-1, anti-NQO-1, anti-UGT1A1, anti-β-actin, and anti-lamin A antibodies were obtained from Santa Cruz Biotechnology. The primary anti-DNMT (DNMT1, DNMT3a, and DNMT3b) antibodies were obtained from IMGENEX and the primary anti-HDAC (HDAC1, HDAC2, HDAC3, and HDAC4) antibodies were obtained from Cell Signaling.

Published OnlineFirst January 17, 2014; DOI: 10.1158/1940-6207.CAPR-13-0313-T

RNA isolation and quantitative real-time PCR

Total RNA was extracted from the treated cells using TRizol reagent (Invitrogen) and the RNeasy Mini Kit (Qiagen). SuperScript III First-Strand cDNA Synthesis System (Invitrogen) was further used to synthesize first-strand cDNA from total RNA according to the manufacturer's instructions. The mRNA expression of specific genes was then determined by quantitative real-time PCR (qPCR) using the total first-strand cDNA as the template and Power SYBR Green PCR Master Mix (Applied Biosystems). The primer pairs used were described previously (22), and the β-actin mRNA expression level was utilized as an internal loading control.

Anchorage-independent cell growth assay

The pretreated cells (8×10^3/well) were transferred to 1 mL of BME containing 0.33% agar over 3 mL of BME containing 0.5% agar with 10% FBS in 6-well plates. The cells were maintained with TPA (20 ng/mL) alone or a combination of TPA (20 ng/mL) and various concentrations of SFN in a 5% CO_2 incubator at 37°C for 14 days. The cell colonies in soft agar were photographed using a computerized microscope system with the Nikon ACT-1 program (Version 2.20; LEAD Technologies) and counted using the ImageJ program (Version 1.40g; NIH).

DNA isolation and bisulfite genomic sequencing

Genomic DNA was isolated from the treated cells using the QIAamp DNA Mini Kit (Qiagen). Bisulfite conversion of genomic DNA was performed using the EZ DNA Methylation Gold Kit (Zymo Research Corp.) according to the manufacturer's instructions, as described previously (12). The DNA fragment containing the first 15 CpGs, which are located between −863 and −1226 in the murine Nrf2 gene with the translation start site defined as position +1, was amplified from the converted DNA by PCR using Platinum Taq DNA polymerase (Invitrogen). The primer sequences were as follows: sense, 5′-AGT TAT GAA GTA GTA GTA AAA A-3′; anti-sense, 5′-ACC CCA AAA AAA TAA ATA AAT C-3′. The PCR products were further cloned into a pCR4 TOPO vector using the TOPO TA Cloning Kit (Invitrogen). At least 10 colonies from each treatment group were randomly selected; the plasmids were prepared using a QIAprep Spin Miniprep Kit (Qiagen) and analyzed by sequencing (GeneWiz).

HDAC activity assay

An HDAC activity assay was performed using Epigenase HDAC Activity/Inhibition Direct Assay Kit (Colorimetric; Epigentek) according to the manufacturer's instructions. The nuclear protein fraction from the treated cells was used, and the relative HDAC activity was calculated based on the ratio of the HDAC activity of the SFN treatment group to that of the control group.

Statistical analysis

The data are represented as the mean ± SD. The statistical analyses were performed using an ANOVA followed by Student *t* test. Statistically significant differences among the means were set at $P < 0.05$ and $P < 0.01$.

Results

SFN increases Nrf2 nuclear translocation and protein expression, upregulating the mRNA and protein expression of Nrf2 target enzymes in JB6 P+ cells

After 5 days of treatment, SFN at 2.5 and 5.0 μmol/L significantly increased the nuclear translocation of the Nrf2 protein in JB6 P+ cells compared with the control group (Fig. 1A). Nrf2 plays an important role in regulating the expression of phase II detoxifying and antioxidative stress enzymes (29). The expression of enzymes regulated by Nrf2 in the JB6 P+ cells treated with SFN for 5 days was measured using qPCR, and the results showed that SFN treatment increased the mRNA expression of HO-1, NQO1, and UGT1A1 in a concentration-dependent manner (Fig. 1B). The protein levels of Nrf2, HO-1, NQO1, and UGT1A1 in the JB6 P+ cells treated with SFN were further evaluated by Western blotting. SFN also increased the protein expression of Nrf2, HO-1, and NQO1 in a concentration-dependent manner; however, UGT1A1 expression was only slightly increased (Fig. 1C). These results suggest that SFN has the potential to increase the Nrf2-mediated mRNA and protein expression of antioxidant and detoxifying enzymes, which might be correlated with the increased cellular expression and nuclear translocation of Nrf2 in JB6 P+ cells.

SFN inhibits TPA-induced JB6 P+ cell transformation

The effect of SFN treatment on the TPA-induced anchorage-independent growth of JB6 P+ cells was examined in soft agar (Fig. 2). The JB6 P+ cells were pretreated with SFN for 5 days and subsequently incubated with TPA with or without SFN for an additional 14 days for the induction of transformation. The results showed that pretreatment with 2.5 or 5.0 μmol/L SFN significantly suppressed the TPA-induced colony formation of the JB6 P+ cells by approximately 55% ($P < 0.05$) and 66% ($P < 0.01$), respectively. When incubated without SFN in soft agar containing TPA for an additional 14 days, SFN (5.0 μmol/L) pretreatment also significantly decreased the number of JB6 P+ colonies compared with the TPA-treated control ($P < 0.01$). These results indicate that SFN has novel chemopreventive potential against TPA-induced carcinogenesis in JB6 P+ cells.

Knockdown of Nrf2 decreases SFN-induced protein expression of Nrf2 and Nrf2 target enzymes

The efficiency of short hairpin RNA (shRNA) knockdown was examined, as shown in Fig. 3, and the protein expression of Nrf2 was significantly decreased after transfection of JB6 cells with shNrf2 in the absence of SFN treatment ($P < 0.01$). However, application of 5.0 μmol/L SFN significantly induced Nrf2 and HO-1 protein expression compared with the control JB6-shMock cells ($P < 0.05$); in contrast, treatment with 1.0 to 5.0 μmol/L SFN only caused a slight increase in Nrf2 and HO-1 expression in the JB6-shNrf2 cells. The induced levels of NQO1 protein in the JB6-shMock

Published OnlineFirst January 17, 2014; DOI: 10.1158/1940-6207.CAPR-13-0313-T

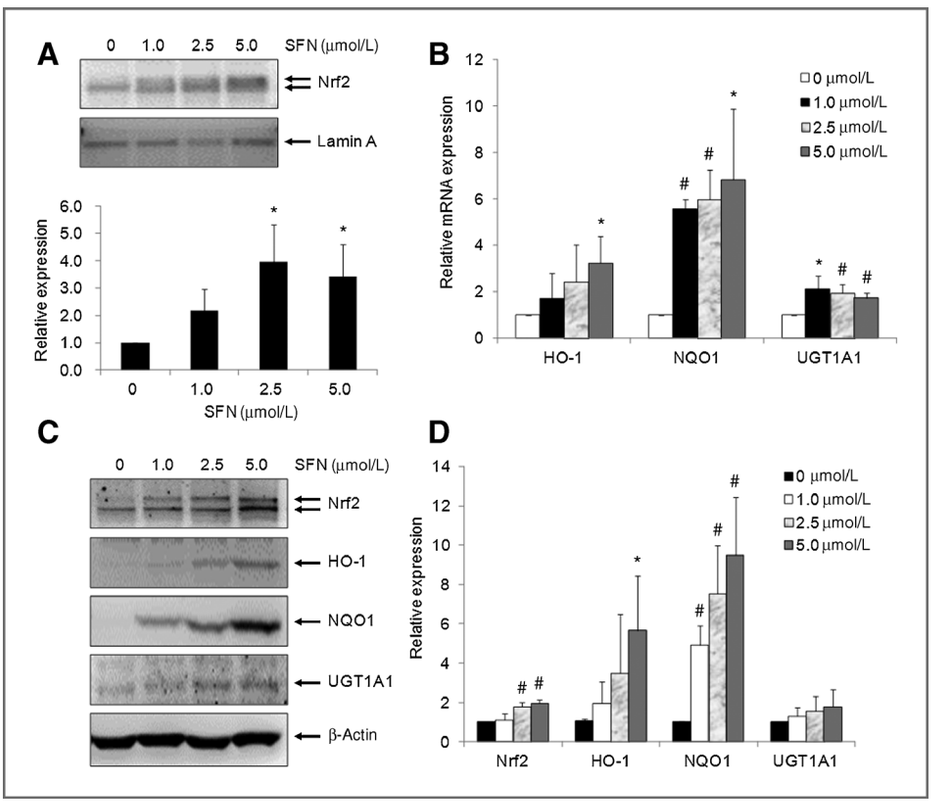

Figure 1. Effect of SFN on Nrf2 nuclear translocation, Nrf2 protein expression, and the mRNA and protein expression of Nrf2 target genes (HO-1, NQO1, and UGT1A1) in JB6 P+ cells. Cells (1×10^5/10-cm dish) were incubated with various concentrations of SFN (0–5.0 μmol/L) for 5 days. A, SFN enhanced the nuclear translocation of Nrf2; the nuclear fraction was isolated as described in Materials and Methods. B, SFN increased the relative transcriptional levels of Nrf2 downstream enzymes. Total mRNA was isolated and analyzed to determine the levels of HO-1, NQO1, and UGT1A1 expression using real-time qPCR after SFN treatment. C, SFN increased the protein expression of Nrf2 and Nrf2 downstream enzymes. Cellular proteins resolved by SDS-PAGE and Western blotting were detected by hybridization with specific antibodies, as described in Materials and Methods. The relative protein expression levels were quantified based on the signal intensity of the corresponding bands from 3 independent experiments and normalized using either lamin A for Nrf2 nuclear protein or β-actin for the total cellular protein level. The graphical data are presented as the mean ± SD from 3 independent experiments. * and # represent $P < 0.05$ and $P < 0.01$, respectively, which indicate significant differences in each target protein or mRNA compared with its level in non-SFN-treated cells.

cells were higher than those in the JB6-shNrf2 cells after treatment with SFN (1.0–5.0 μmol/L). These results indicate that SFN might inhibit cell transformation in JB6 P+ cells through the upregulation of cellular Nrf2, resulting in an increase in the protein levels of Nrf2 downstream genes, including HO-1 and NQO1.

Knockdown of Nrf2 increases the sensitivity of JB6 P+ cells to TPA-induced cell transformation, a state that is resistant to the protective effect of SFN

A stable Nrf2-knockdown JB6 P+ cell line was established using a shRNA to evaluate whether the resistance of the cells to TPA-induced cell transformation is directly correlated with the status of Nrf2 in the cells. Knocking down Nrf2 significantly increased the growth of the JB6-shNrf2 cells in soft agar compared with the JB6-shMock cells ($P < 0.01$; Fig. 4). Moreover, pretreatment with SFN (1.0–5.0 μmol/L) significantly inhibited the TPA-induced anchorage-independent growth of the JB6-shMock cells by approximately 28% to 56%. In contrast, this SFN-mediated inhibition was reduced in the JB6-shNrf2 cells, indicating that the protective effect of SFN in JB6-shNrf2 cells was decreased. These results suggest that Nrf2 plays an important role in the preventive effect of SFN on TPA-induced JB6 P+ cell transformation.

SFN increases relative Nrf2 mRNA expression when cells are treated with TPA and decreases the methylation status of the Nrf2 gene promoter

The expression of Nrf2 and its downstream genes has previously been shown to be decreased by SFN in the tumorigenic TRAMP C1 cell line via the epigenetic activation of the Nrf2 gene (28). In this study, JB6 P+ cells were pretreated with SFN for 5 days and subsequently incubated with TPA for an additional 6 hours. The mRNA expression level of Nrf2 was then measured. The results indicated that pretreatments with both 2.5 and 5.0 μmol/L SFN increased the ratio of relative Nrf2 mRNA expression (Fig. 5A). To examine whether SFN pretreatment caused demethylation of the promoter region resulting in increased Nrf2 transcription, bisulfite sequencing was performed to investigate

Published OnlineFirst January 17, 2014; DOI: 10.1158/1940-6207.CAPR-13-0313-T

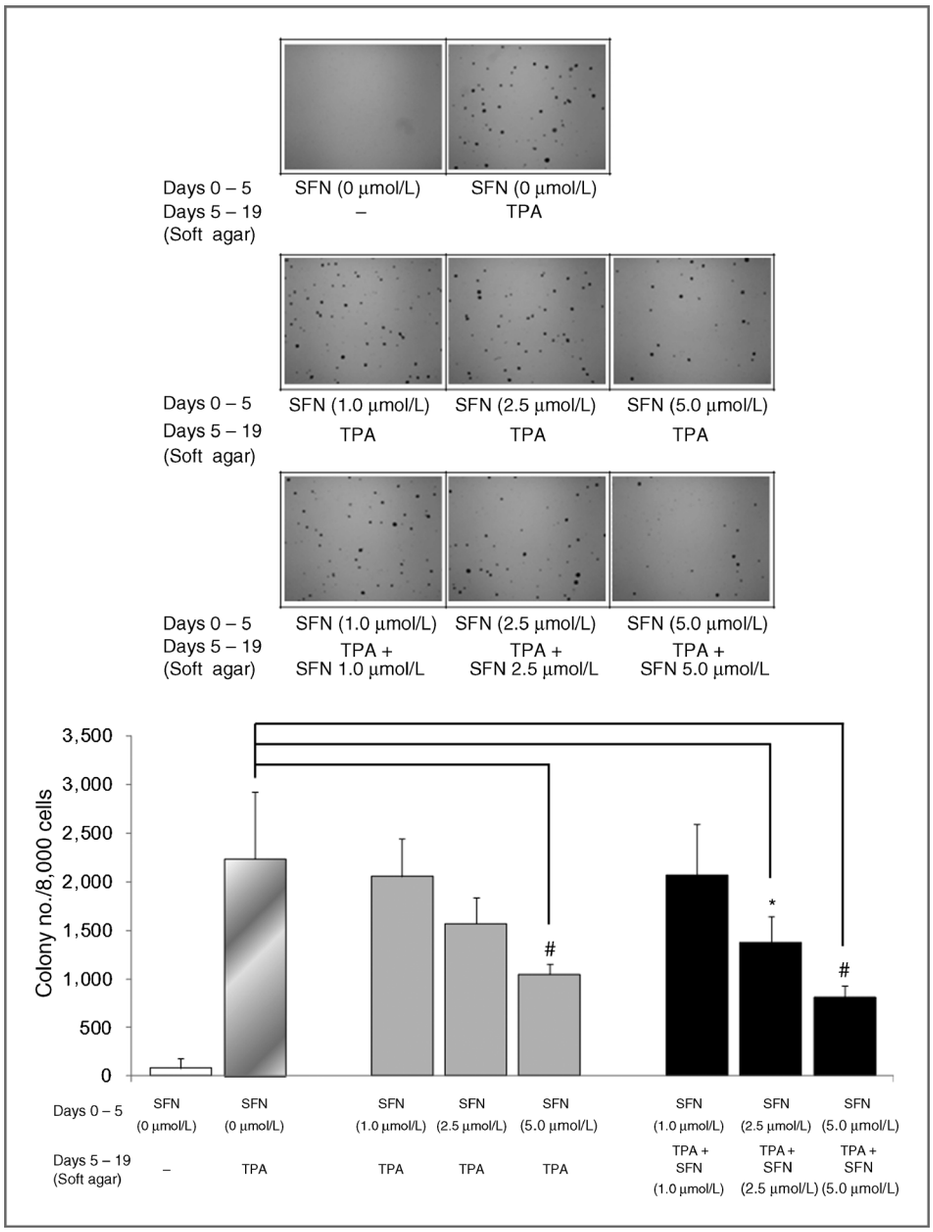

Figure 2. Inhibitory effects of SFN on the TPA-induced transformation of JB6 P+ cells. Cells (1×10^5/10-cm dish) were treated with SFN (0–5.0 μmol/L) for 5 days. The pretreated cells (at a density of 8,000 cells/well) were then transferred to soft agar containing TPA with or without SFN in 6-well plates for an additional 14 days. The colonies exhibiting anchorage-independent growth were counted under a microscope using ImageJ software. The data are represented as the average of triplicate results from 2 independent experiments. * and # represent $P < 0.05$ and $P < 0.01$, respectively, which indicate significant differences between the SFN-treated group and cells treated with TPA alone in soft agar.

the methylation status of the first 15 CpGs of Nrf2, similar to our previous study in TRAMP C1 cells (12). Figure 5B shows that the 15 CpGs were hypermethylated in the control group (methylation ratio, 88.0%). The ratio of methylated CpGs was decreased to 68.7% by SFN (2.5 μmol/L), whereas 5-aza (250 nmol/L)/TSA (50 nmol/L) reduced the methylation level to 50.0%. These data suggest that SFN can demethylate the Nrf2 gene promoter in JB6 P+ cells.

SFN downregulates epigenetic modifying enzymes in JB6 P+ cells

The effects of SFN on epigenetic modifying enzymes, including DNMTs and HDACs, were further examined to explore the epigenetic mechanism of SFN in promoter demethylation and the induction of Nrf2 gene transcription. We found that SFN (1.0–5.0 μmol/L) decreased the protein expression of DNMT1, DNMT3a, and DNMT3b in a concentration-dependent manner in JB6 P+ cells after 5 days of treatment (Fig. 6). In addition, treatment with either 2.5 or 5.0 μmol/L SFN significantly inhibited the relative HDAC activity by 50% ($P < 0.01$; Fig. 7A), which might be because of the reduced level of HDAC1, HDAC2, HDAC3, and HDAC4 protein expression caused by SFN treatment (Fig. 7B).

Discussion

Carcinogenesis is caused by a cumulative, multistage process that mainly consists of initiation, promotion, and

Published OnlineFirst January 17, 2014; DOI: 10.1158/1940-6207.CAPR-13-0313-T

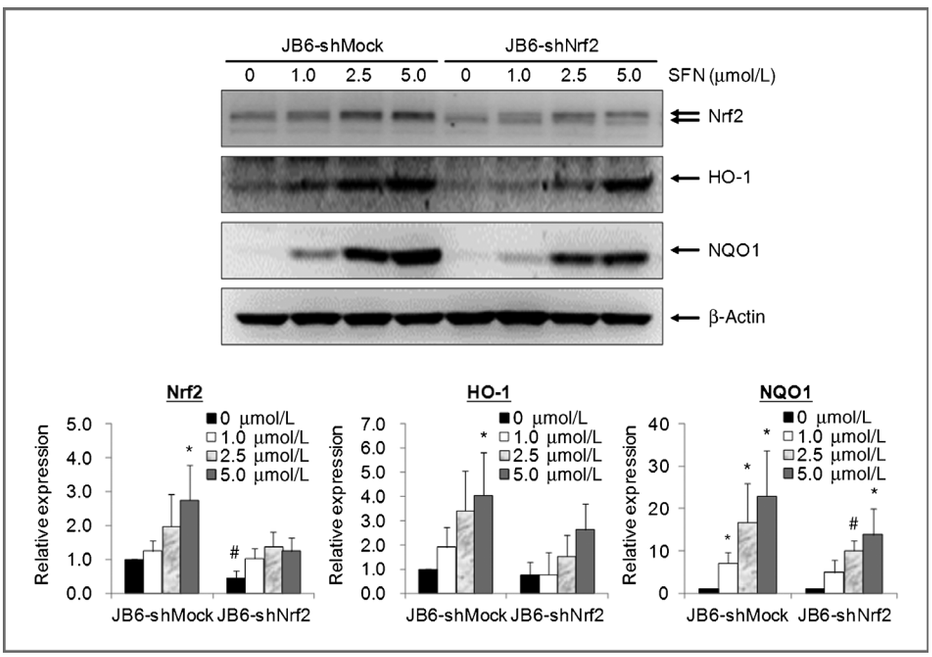

Figure 3. Effect of SFN on the protein expression of Nrf2, HO-1, and NQO1 in JB6-shMock and JB6-shNrf2 cells. Cells (1×10^5/10-cm dish) were incubated with various concentrations of SFN (0–5.0 μmol/L) for 5 days. Separation of the cellular proteins was achieved using SDS-PAGE and Western blotting, as described in the Materials and Methods. The protein expression of Nrf2 and the Nrf2 target enzymes HO-1 and NQO1 was detected by hybridization with specific antibodies. The relative expression levels were quantified based on the signal intensity of the corresponding bands from 3 independent experiments and were normalized using β-actin. The graphical data are presented as the mean ± SD from 3 independent experiments. * and # represent $P < 0.05$ and $P < 0.01$, respectively, which indicate significant differences in the target proteins compared with their levels in JB6-shMock cells without SFN treatment.

progression. ROS, which are produced as a result of the metabolism of molecular oxygen via biochemical reactions in cells, play a key role in tumor promotion. Some tumor promoters accelerate/induce the conversion of initiated cells (carcinogen-mediated mutation or potential "stem cells") into tumorigenic cells possibly via the production of oxidative/inflammatory responses (30, 31). TPA (12-*O*-tetradecanoylphorbol-13-acetate), a phorbol ester, is a tumor promoter that induces the neoplastic/tumorigenic transformation of preneoplastic JB6 cells through the overproduction of ROS (32). In this study, we investigated the inhibitory effect of SFN on TPA-stimulated neoplastic transformation in the mouse epidermal JB6 P+ cell line to assess whether SFN is able to block tumor promoter-induced tumorigenesis in skin cells. Our results show that SFN was effective when it was given together with the tumor promoter TPA as well as when it was given before TPA that would have produced some biologic responses resulting in subsequent inhibition of transformation of JB6 P+ cells by TPA (Fig. 2). Pretreatment with SFN would increase the protein and mRNA expression of HO-1, NQO1, and UGT1A1 (Fig. 1B and C), among others, that could potentially explain the inhibitory effects on transformation. The multiple mechanisms of SFN in the induction of Nrf2 were subsequently been demonstrated, including increased protein expression and nuclear translocation of Nrf2 and enhanced the transcription level of Nrf2 by epigenetic regulation.

Nrf2 has been identified as an important transcription factor to regulate the expression of phase II antioxidant and detoxification enzymes, thus maintaining the balance of cellular ROS (6, 33). The transcriptional activation of antioxidant and detoxification genes is increased by Nrf2 through its interaction with AREs (34), and accumulating evidence indicates that dietary phytochemicals with chemopreventive potential could enhance the activities of antioxidant and detoxification enzymes via the Nrf2 pathway (35). This study shows that SFN increased HO-1, NQO1, and UGT1A1 mRNA and protein levels as well as the nuclear translocation and expression of total endogenous Nrf2 protein in JB6 P+ cells (Fig. 1). Keap1, a negative regulator of Nrf2, is a primary redox sensor with novel sulfhydryl residues that are sensitive to electrophiles and ROS, and Nrf2 itself may regulate its subcellular localization through its MES_{TA} motif (33, 36). Interestingly, it has been reported that SFN can modify multiple Keap1 domains and form thionoacyl adducts (37). The SFN-induced thiocarbamylation at cysteine 151 of Keap1 may disrupt the interaction between Keap1 and Nrf2, leading to the cytoplasmic and nuclear accumulation of Nrf2 (38–40). Therefore, the interaction between SFN and Keap1 is considered to be a potential mechanism for the induction of Nrf2 in JB6 P+ cells.

The chemopreventive efficacy of SFN against tumorigenesis via a critical Nrf2-mediated pathway has been demonstrated in several animal models, such as DMBA/TPA-induced skin carcinogenesis in Nrf2-deficient C57BL/6

Published OnlineFirst January 17, 2014; DOI: 10.1158/1940-6207.CAPR-13-0313-T

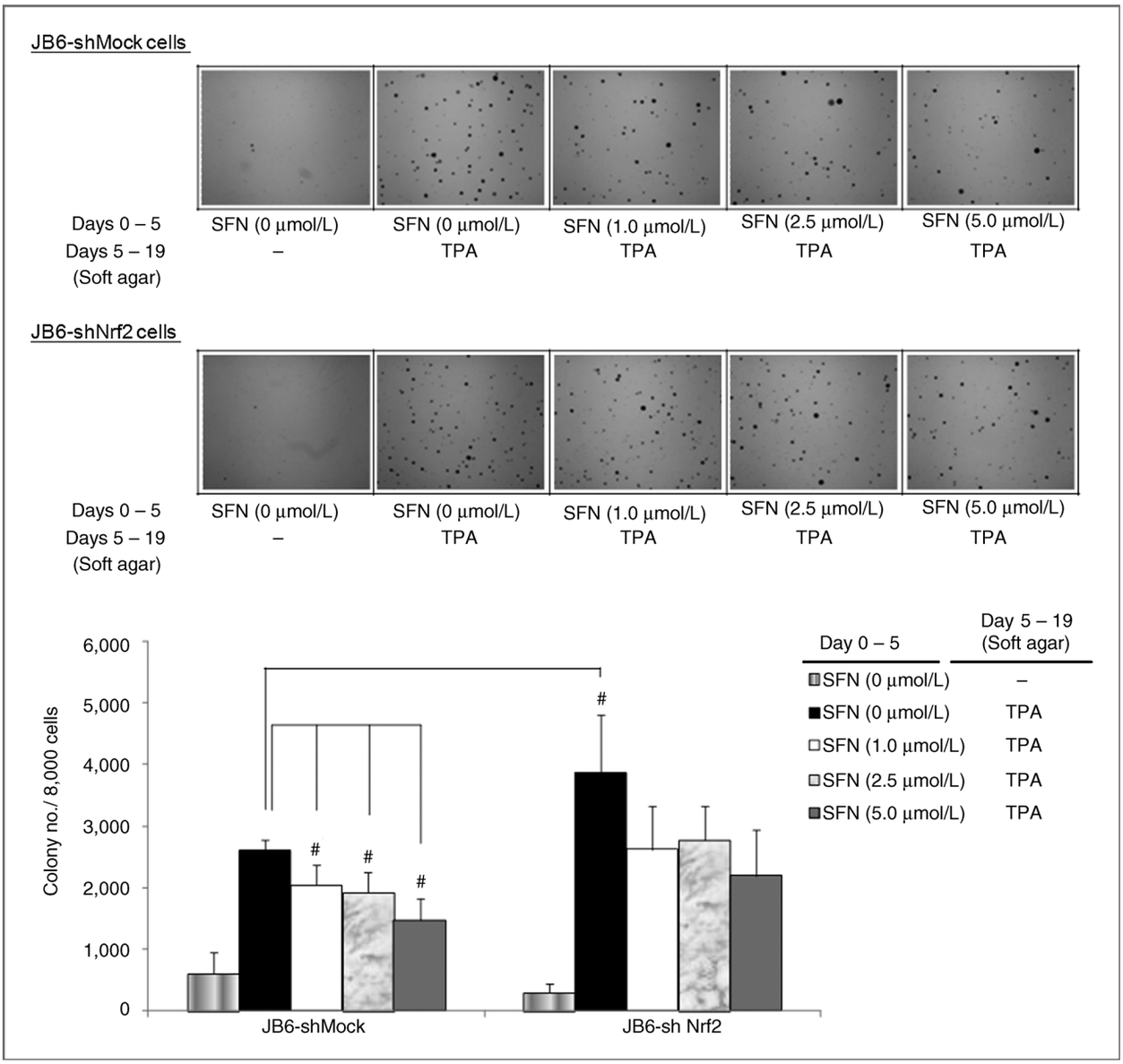

Figure 4. Inhibitory effect of SFN pretreatment on the TPA-induced transformation of shMock- and shNrf2-transfected JB6 P+ cells. Cells (1×10^5/10-cm dish) were treated with SFN (0–5.0 μmol/L) for 5 days. The pretreated cells (at a density of 8,000 cells/well) were then transferred to soft agar containing TPA in 6-well plates for an additional 14 days. The colonies exhibiting anchorage-independent growth were counted under a microscope using ImageJ software. The graphical data are presented as the average of triplicate results from 2 independent experiments. #, $P < 0.01$ indicating a significant decrease or increase in colony formation compared with the JB6-shMock cells treated with TPA alone in soft agar.

mice (25) and benzo[*a*]pyrene-induced fore-stomach tumors in Nrf2-deficient ICR mice (41). In our previous study using TRAMP mice, broccoli sprouts, which contain abundant SFN, were found to suppress prostate tumorigenesis via the activation of the Nrf2 pathway (42). We have also used oligonucleotide microarray analysis to compare gene expression profiles between Nrf2-knockout mice and wild-type mice administered SFN via oral gavage; through these experiments, we identified Nrf2-dependent SFN-inducible antioxidant and detoxification genes (43). In this study, the critical role of Nrf2 induction via SFN pretreatment for the prevention of TPA-induced neoplastic transformation of JB6 P+ cells was demonstrated using a stable Nrf2-knockdown cell line (Fig. 4). In this line, reduced expression of the Nrf2 downstream enzymes HO-1 and NQO1 was induced by SFN (Fig. 3).

Epigenetic regulation, including DNA methylation and histone modification, is an important process in cancer development, and the epigenetic regulatory activity of certain dietary phytochemicals with cancer chemopreventive potential has been demonstrated, including curcumin (21), EGCG (20), and genistein (44). We have previously shown

Published OnlineFirst January 17, 2014; DOI: 10.1158/1940-6207.CAPR-13-0313-T

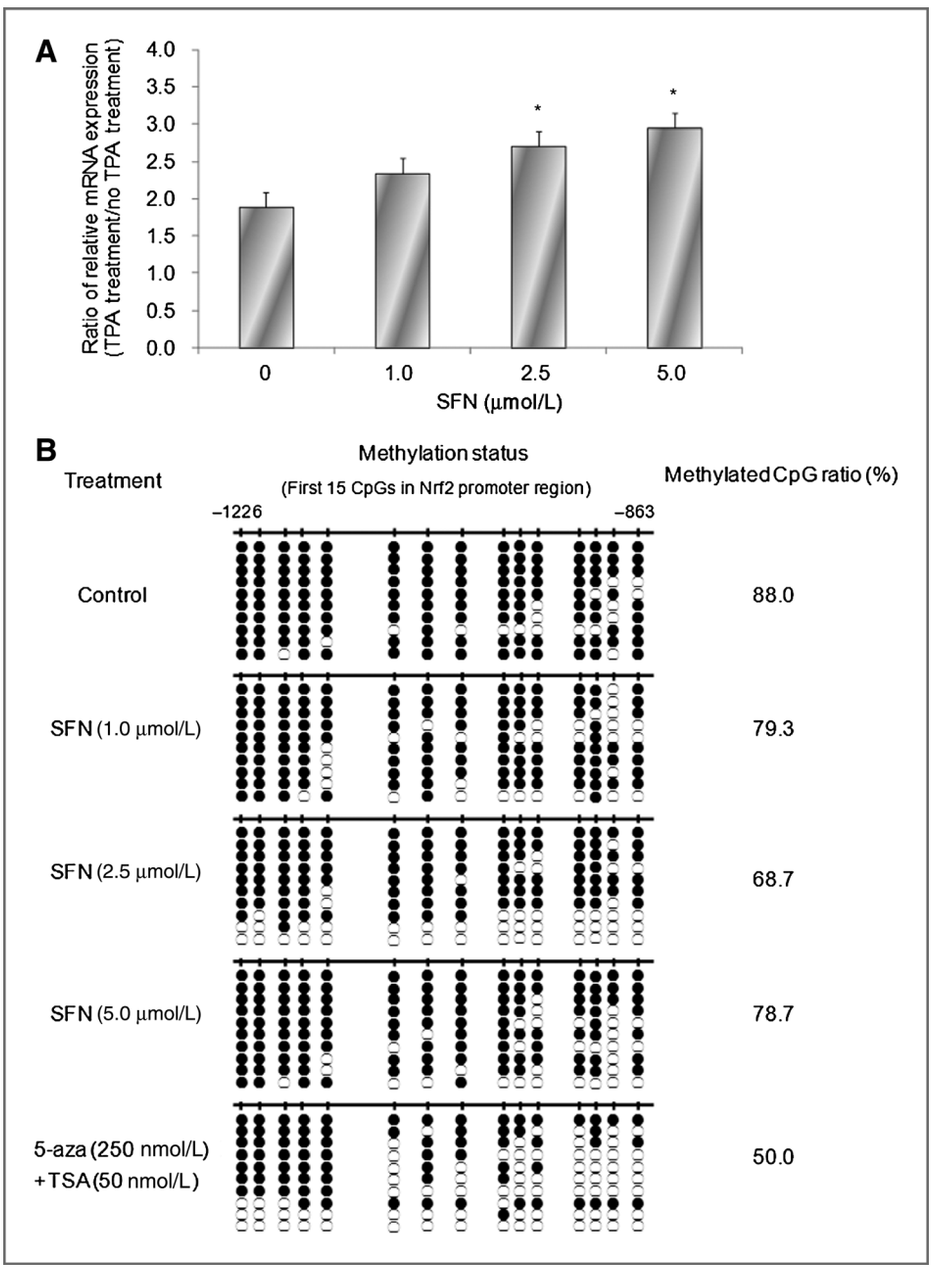

Figure 5. Effect of SFN on the Nrf2 mRNA expression induced by TPA and methylation alteration of the Nrf2 promoter regions in JB6 P+ cells. A, pretreatment of SFN increased the relative amount of mRNA expression of Nrf2 in the cells exposed to TPA. Cells (1.5×10^4/6-cm dish) incubated with various concentrations of SFN (0–5.0 μmol/L) for 5 days were further exposed to TPA for an additional 6 hours, and total mRNA was isolated and analyzed for Nrf2 mRNA expression using real-time qPCR. *, $P < 0.05$ indicating a significant difference in mRNA expression compared with non-SFN-treated cells. B, genomic DNA was extracted from SFN-treated cells (1×10^5/10-cm dish) after 5 days of treatment. For the 5-aza (250 nmol/L)/TSA (50 nmol/L) combination treatment, TSA was added as a positive control at 20 hours before harvesting. The methylation patterns of the first 15 CpGs, which are located at −1226 to −863 from the translational start site (labeled as +1) in the promoter of the Nrf2 gene, were determined using bisulfite genomic sequencing, as described in Materials and Methods. The filled and open dots indicate methylated and unmethylated CpGs, respectively. Methylated CpG ratio: the percentage of methylated CpGs was based on the total CpGs in each treatment group.

that the expression of Nrf2 can be attenuated by DNA CpG methylation in both the prostate tumor tissue of TRAMP mice and tumorigenic TRAMP C1 cells (12). Subsequently some effective cancer chemopreventive compounds were found to demethylate the CpG of Nrf2 promoter with the resulting increased of Nrf2 expression in *in vivo* (TRAMP mice) or *in vitro* (TRAMP C1 cells) studies (21, 22, 28, 45, 46). These studies suggest that the potent chemopreventive compounds that epigenetically restore the silenced Nrf2 gene via DNA demethylation could be used as epigenetic modifying cancer chemopreventive and therapeutic agents. Our present study provides further evidence about the demethylation activity of SFN on the first 15 CpGs of the Nrf2 promoter in JB6 P+ cells, and we show that the transcription of a hypomethylated Nrf2 gene might be more easily induced upon exposure to TPA (Fig. 5). These results also suggest that DNA demethylation is associated with Nrf2 activation in SFN-treated JB6 cells corroborating with previous published findings that DNA CpG methylation in the promoter of Nrf2 gene could serve as a potential key factor for Nrf2 gene silencing.

Epigenetic modification enzymes, such as DNMTs and HDACs that silence gene expression, are the major targets of cancer prevention and therapeutic strategies. Previous reports indicate that SFN can suppress DNMT1 and DNMT3a in TRAMP C1 cells (28) and human breast cancer cells (47),

Published OnlineFirst January 17, 2014; DOI: 10.1158/1940-6207.CAPR-13-0313-T

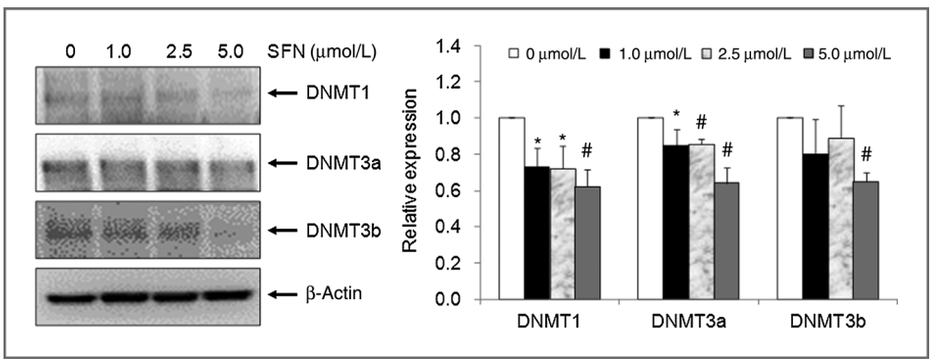

Figure 6. Effect of SFN on DNMT protein expression in JB6 P+ cells. Cells (1×10^5/10-cm dish) were incubated with various concentrations of SFN (0–5.0 μmol/L) for 5 days, and the proteins were resolved by SDS-PAGE and Western blotting, as described in Materials and Methods. The expression of DNMT1, DNMT3a, and DNMT3b proteins was detected by hybridization with specific antibodies. The relative expression levels were quantified based on the signal intensity of the corresponding bands and normalized using β-actin. The graphical data are represented as the mean ± SD from 3 independent experiments. * and # represent $P < 0.05$ and $P < 0.01$, respectively, which indicate significant differences in each target protein compared with its level in non-SFN-treated cells.

which may result in hypomethylation of the Nrf2 promoter and the first exon of the hTERT gene, respectively. We also found that the expression of the DNMT1, DNMT3a, and DNMT3b proteins was decreased in JB6 P+ cells after treatment with SFN (Fig. 6). In addition to DNMTs, the inhibitory effect of SFN on HDAC has been demonstrated in prostate epithelial BPH-1, LnCaP, and PC-3 cells (48). In addition to SFN, 2 major metabolites, SFN-cysteine and SFN-*N*-acetyl-cysteine, can suppress HDAC activity *in vitro* (49). In an *in vivo* study, the SFN-induced decrease in HDAC activity suppressed the growth of PC-3 xenografts, and SFN-rich broccoli sprouts inhibited HDAC activity in peripheral blood mononuclear cells in human subjects (50). SFN also causes histone modification in the colonic mucosa and polyps of Apc-minus mice through inhibition of HDAC activity, which might retard intestinal carcinogenesis (51). Histone modification was also observed in MCF-7 and MDA-MB-231 cells after SFN treatment, including an increase in active chromatin markers (acetyl-H3, acetyl-H3K9, and acetyl-H4) and a decrease in inactive chromatin markers (trimethyl-H3K9 and trimethyl-H3K27; ref. 47). Our present results show that the total activity of HDACs and the protein levels of HDAC1, HDAC2, HDAC3, and HDAC4 were significantly decreased by SFN in a dose-dependent manner (Fig. 7), suggesting that transcriptional activity of the Nrf2 gene may also be regulated by SFN through histone modification.

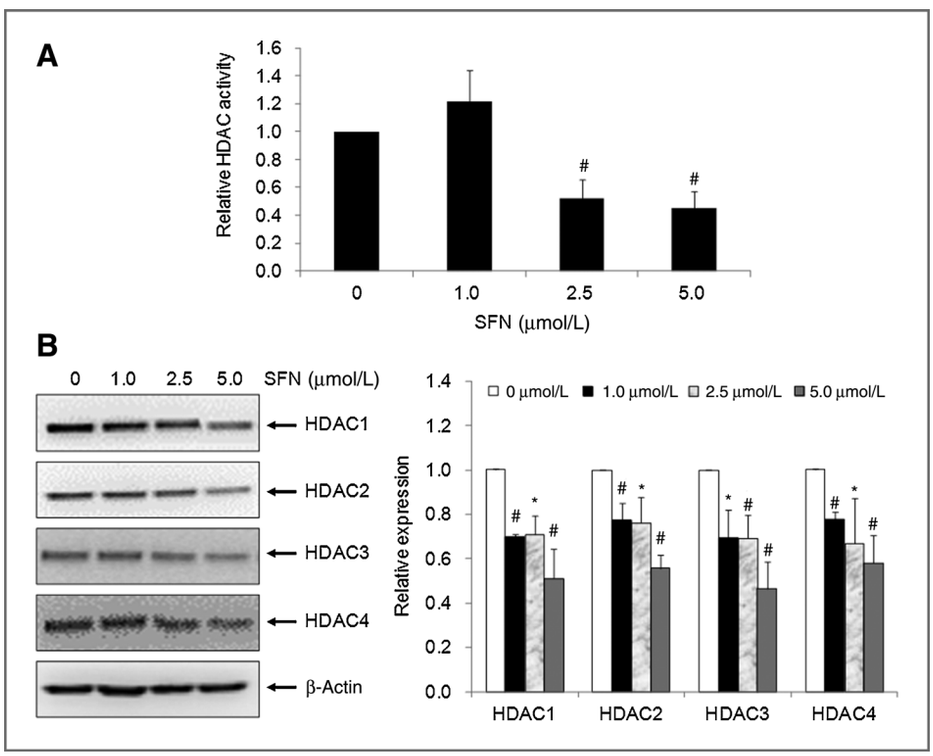

Figure 7. Effect of SFN on the total activity and protein expression of HDACs in JB6 P+ cells. Cells (1×10^5/10-cm dish) were incubated with various concentrations of SFN (0–5.0 μmol/L) for 5 days. A, SFN inhibited total HDAC activity; the nuclear fraction was isolated and analyzed for total HDAC activity, as described in Materials and Methods. B, SFN decreased HDAC protein expression. The proteins were resolved by SDS-PAGE and Western blotting, as described in Materials and Methods. The expression of HDAC1, HDAC2, HDAC3, and HDAC4 proteins were detected by hybridization with specific antibodies. The relative expression levels were quantified based on the signal intensity of the corresponding bands and normalized using β-actin. The graphical data are presented as the mean ± SD from 3 independent experiments. * and # represent $P < 0.05$ and $P < 0.01$, respectively, which indicate significant differences in each target protein compared with its level in non-SFN-treated cells.

Published OnlineFirst January 17, 2014; DOI: 10.1158/1940-6207.CAPR-13-0313-T

In conclusion, our present study shows that SFN induces the nuclear translocation of Nrf2, resulting in enhanced Nrf2-mediated expression of the cellular defense enzymes HO-1 and NQO1. Furthermore, our epigenetic analysis shows that SFN inhibits DNMT and HDAC protein expression, attenuates total HDAC activity, and decreases methylation of the first 15 CpGs of the Nrf2 promoter, resulting in enhanced Nrf2 mRNA and protein expression. Collectively, these results suggest that the SFN-mediated antineoplastic transformation of JB6 P+ cells induced by the tumor promoter TPA may involve multiple molecular mechanisms, including epigenetic reprogramming, Nrf2 reactivation, and enhanced expression of Nrf2 and Nrf2 target genes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Z.-Y. Su, C. Zhang, T.O. Khor, A.H. Conney, A.-N.T. Kong
Development of methodology: Z.-Y. Su, C. Zhang, L. Shu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z.-Y. Su, C. Zhang, J.H. Lee, T.-Y. Wu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z.-Y. Su, C. Zhang, T.-Y. Wu
Writing, review, and/or revision of the manuscript: Z.-Y. Su, A.H. Conney, Y.-P. Lu, A.-N.T. Kong
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.H. Lee, A.H. Conney, A.-N.T. Kong
Study supervision: T.O. Khor, A.H. Conney, A.-N.T. Kong

Acknowledgments

The authors thank all the members in Dr. A.-N.T. Kong's lab for their helpful discussion and preparation of this manuscript.

Grant Support

This work was supported by institutional funds of Rutgers, The State University of New Jersey.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 30, 2013; revised November 27, 2013; accepted December 16, 2013; published OnlineFirst January 17, 2014.

References

1. Federico A, Morgillo F, Tuccillo C, Ciardiello F, Loguercio C. Chronic inflammation and oxidative stress in human carcinogenesis. Int J Cancer 2007;121:2381–6.
2. Trouba KJ, Hamadeh HK, Amin RP, Germolec DR. Oxidative stress and its role in skin disease. Antioxid Redox Signal 2002;4:665–73.
3. Kobayashi A, Ohta T, Yamamoto M. Unique function of the Nrf2-Keap1 pathway in the inducible expression of antioxidant and detoxifying enzymes. Methods Enzymol 2004;378:273–86.
4. Kwak MK, Wakabayashi N, Kensler TW. Chemoprevention through the Keap1-Nrf2 signaling pathway by phase 2 enzyme inducers. Mutat Res 2004;555:133–48.
5. Cullinan SB, Gordan JD, Jin J, Harper JW, Diehl JA. The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase. Mol Cell Biol 2004;24:8477–86.
6. Chen C, Kong AN. Dietary chemopreventive compounds and ARE/EpRE signaling. Free Radic Biol Med 2004;36:1505–16.
7. Nguyen T, Nioi P, Pickett CB. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. J Biol Chem 2009;284:13291–5.
8. Ramos-Gomez M, Kwak MK, Dolan PM, Itoh K, Yamamoto M, Talalay P, et al. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. Proc Natl Acad Sci U S A 2001;98:3410–5.
9. Khor TO, Huang MT, Prawan A, Liu Y, Hao X, Yu S, et al. Increased susceptibility of Nrf2 knockout mice to colitis-associated colorectal cancer. Cancer Prev Res 2008;1:187–91.
10. Hu R, Saw CL, Yu R, Kong AN. Regulation of NF-E2-related factor 2 signaling for cancer chemoprevention: antioxidant coupled with anti-inflammatory. Antioxid Redox Signal 2010;13:1679–98.
11. Kwak MK, Egner PA, Dolan PM, Ramos-Gomez M, Groopman JD, Itoh K, et al. Role of phase 2 enzyme induction in chemoprotection by dithiolethiones. Mutat Res 2001;480–481:305–15.
12. Yu S, Khor TO, Cheung KL, Li W, Wu TY, Huang Y, et al. Nrf2 expression is regulated by epigenetic mechanisms in prostate cancer of TRAMP mice. PLoS ONE 2010;5:e8579.
13. Esteller M. Epigenetics in cancer. N Engl J Med 2008;358:1148–59.
14. Devinoy E, Rijnkels M. Epigenetics in mammary gland biology and cancer. J Mammary Gland Biol Neoplasia 2010;15:1–4.
15. Kim TY, Bang YJ, Robertson KD. Histone deacetylase inhibitors for cancer therapy. Epigenetics 2006;1:14–23.
16. Walton TJ, Li G, Seth R, McArdle SE, Bishop MC, Rees RC. DNA demethylation and histone deacetylation inhibition co-operate to re-express estrogen receptor beta and induce apoptosis in prostate cancer cell-lines. Prostate 2008;68:210–22.
17. Hauser AT, Jung M. Targeting epigenetic mechanisms: potential of natural products in cancer chemoprevention. Planta Med 2008;74:1593–601.
18. Meeran SM, Ahmed A, Tollefsbol TO. Epigenetic targets of bioactive dietary components for cancer prevention and therapy. Clin Epigenetics 2010;1:101–16.
19. Fang MZ, Wang Y, Ai N, Hou Z, Sun Y, Lu H, et al. Tea polyphenol (−)-epigallocatechin-3-gallate inhibits DNA methyltransferase and reactivates methylation-silenced genes in cancer cell lines. Cancer Res 2003;63:7563–70.
20. Pandey M, Shukla S, Gupta S. Promoter demethylation and chromatin remodeling by green tea polyphenols leads to re-expression of GSTP1 in human prostate cancer cells. Int J Cancer 2010;126:2520–33.
21. Khor TO, Huang Y, Wu TY, Shu L, Lee J, Kong AN. Pharmacodynamics of curcumin as DNA hypomethylation agent in restoring the expression of Nrf2 via promoter CpGs demethylation. Biochem Pharmacol 2011;82:1073–8.
22. Su ZY, Khor TO, Shu L, Lee JH, Saw CL, Wu TY, et al. Epigenetic reactivation of Nrf2 in murine prostate cancer TRAMP C1 cells by natural phytochemicals Z-ligustilide and Radix angelica sinensis via promoter CpG demethylation. Chem Res Toxicol 2013;26:477–85.
23. Cheung KL, Kong AN. Molecular targets of dietary phenethyl isothiocyanate and sulforaphane for cancer chemoprevention. AAPS J 2010;12:87–97.
24. Dinkova-Kostova AT, Jenkins SN, Fahey JW, Ye L, Wehage SL, Liby KT, et al. Protection against UV-light-induced skin carcinogenesis in SKH-1 high-risk mice by sulforaphane-containing broccoli sprout extracts. Cancer Lett 2006;240:243–52.
25. Xu C, Huang MT, Shen G, Yuan X, Lin W, Khor TO, et al. Inhibition of 7,12-dimethylbenz(a)anthracene-induced skin tumorigenesis in C57BL/6 mice by sulforaphane is mediated by nuclear factor E2-related factor 2. Cancer Res 2006;66:8293–6.
26. Abel EL, Boulware S, Fields T, McIvor E, Powell KL, DiGiovanni J, et al. Sulforaphane induces phase II detoxication enzymes in mouse skin and prevents mutagenesis induced by a mustard gas analog. Toxicol Appl Pharmacol 2013;266:439–42.
27. Ho E, Clarke JD, Dashwood RH. Dietary sulforaphane, a histone deacetylase inhibitor for cancer prevention. J Nutr 2009;139:2393–6.

Published OnlineFirst January 17, 2014; DOI: 10.1158/1940-6207.CAPR-13-0313-T

28. Zhang C, Su ZY, Khor TO, Shu L, Kong AN. Sulforaphane enhances Nrf2 expression in prostate cancer TRAMP C1 cells through epigenetic regulation. Biochem Pharmacol 2013;85:1398–404.
29. Barve A, Khor TO, Nair S, Reuhl K, Suh N, Reddy B, et al. Gamma-tocopherol-enriched mixed tocopherol diet inhibits prostate carcinogenesis in TRAMP mice. Int J Cancer 2009;124:1693–9.
30. Ito N, Hirose M. Antioxidants—carcinogenic and chemopreventive properties. Adv Cancer Res 1989;53:247–302.
31. Pillai CK, Pillai KS. Antioxidants in health. Indian J Physiol Pharmacol 2002;46:1–5.
32. Dhar A, Young MR, Colburn NH. The role of AP-1, NF-κB and ROS/NOS in skin carcinogenesis: the JB6 model is predictive. Mol Cell Biochem 2002;234–235:185–93.
33. Li W, Kong AN. Molecular mechanisms of Nrf2-mediated antioxidant response. Mol Carcinog 2009;48:91–104.
34. Kang KW, Lee SJ, Kim SG. Molecular mechanism of nrf2 activation by oxidative stress. Antioxid Redox Signal 2005;7:1664–73.
35. Su ZY, Shu L, Khor TO, Lee JH, Fuentes F, Kong AN. A perspective on dietary phytochemicals and cancer chemoprevention: oxidative stress, Nrf2, and epigenomics. Top Curr Chem 2013;329:133–62.
36. Dinkova-Kostova AT, Holtzclaw WD, Wakabayashi N. Keap1, the sensor for electrophiles and oxidants that regulates the phase 2 response, is a zinc metalloprotein. Biochemistry 2005;44:6889–99.
37. Hong F, Freeman ML, Liebler DC. Identification of sensor cysteines in human Keap1 modified by the cancer chemopreventive agent sulforaphane. Chem Res Toxicol 2005;18:1917–26.
38. Kobayashi M, Li L, Iwamoto N, Nakajima-Takagi Y, Kaneko H, Nakayama Y, et al. The antioxidant defense system Keap1-Nrf2 comprises a multiple sensing mechanism for responding to a wide range of chemical compounds. Mol Cell Biol 2009;29:493–502.
39. McMahon M, Lamont DJ, Beattie KA, Hayes JD. Keap1 perceives stress via three sensors for the endogenous signaling molecules nitric oxide, zinc, and alkenals. Proc Natl Acad Sci U S A 2010;107:18838–43.
40. Hu C, Eggler AL, Mesecar AD, van Breemen RB. Modification of keap1 cysteine residues by sulforaphane. Chem Res Toxicol 2011;24:515–21.
41. Fahey JW, Haristoy X, Dolan PM, Kensler TW, Scholtus I, Stephenson KK, et al. Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of Helicobacter pylori and prevents benzo[a]pyrene-induced stomach tumors. Proc Natl Acad Sci U S A 2002;99:7610–5.
42. Keum YS, Khor TO, Lin W, Shen G, Kwon KH, Barve A, et al. Pharmacokinetics and pharmacodynamics of broccoli sprouts on the suppression of prostate cancer in transgenic adenocarcinoma of mouse prostate (TRAMP) mice: implication of induction of Nrf2, HO-1 and apoptosis and the suppression of Akt-dependent kinase pathway. Pharm Res 2009;26:2324–31.
43. Hu R, Xu C, Shen G, Jain MR, Khor TO, Gopalkrishnan A, et al. Gene expression profiles induced by cancer chemopreventive isothiocyanate sulforaphane in the liver of C57BL/6J mice and C57BL/6J/Nrf2 (−/−) mice. Cancer Lett 2006;243:170–92.
44. Majid S, Dar AA, Ahmad AE, Hirata H, Kawakami K, Shahryari V, et al. BTG3 tumor suppressor gene promoter demethylation, histone modification and cell cycle arrest by genistein in renal cancer. Carcinogenesis 2009;30:662–70.
45. Huang Y, Khor TO, Shu L, Saw CL, Wu TY, Suh N, et al. A gamma-tocopherol-rich mixture of tocopherols maintains Nrf2 expression in prostate tumors of TRAMP mice via epigenetic inhibition of CpG methylation. J Nutr 2012;142:818–23.
46. Wu TY, Khor TO, Su ZY, Saw CL, Shu L, Cheung KL, et al. Epigenetic modifications of Nrf2 by 3,3′-diindolylmethane *in vitro* in TRAMP C1 cell line and *in vivo* TRAMP prostate tumors. AAPS J 2013;15:864–74.
47. Meeran SM, Patel SN, Tollefsbol TO. Sulforaphane causes epigenetic repression of hTERT expression in human breast cancer cell lines. PLoS ONE 2010;5:e11457.
48. Myzak MC, Hardin K, Wang R, Dashwood RH, Ho E. Sulforaphane inhibits histone deacetylase activity in BPH-1, LnCaP and PC-3 prostate epithelial cells. Carcinogenesis 2006;27:811–9.
49. Myzak MC, Karplus PA, Chung FL, Dashwood RH. A novel mechanism of chemoprotection by sulforaphane: inhibition of histone deacetylase. Cancer Res 2004;64:5767–74.
50. Myzak MC, Tong P, Dashwood WM, Dashwood RH, Ho E. Sulforaphane retards the growth of human PC-3 xenografts and inhibits HDAC activity in human subjects. Exp Biol Med (Maywood) 2007;232:227–34.
51. Myzak MC, Dashwood WM, Orner GA, Ho E, Dashwood RH. Sulforaphane inhibits histone deacetylase *in vivo* and suppresses tumorigenesis in Apc-minus mice. FASEB J 2006;20:506–8.

Published OnlineFirst January 17, 2014; DOI: 10.1158/1940-6207.CAPR-13-0313-T

Cancer Prevention Research

Requirement and Epigenetics Reprogramming of Nrf2 in Suppression of Tumor Promoter TPA-Induced Mouse Skin Cell Transformation by Sulforaphane

Zheng-Yuan Su, Chengyue Zhang, Jong Hun Lee, et al.

Cancer Prev Res 2014;7:319-329. Published OnlineFirst January 17, 2014.